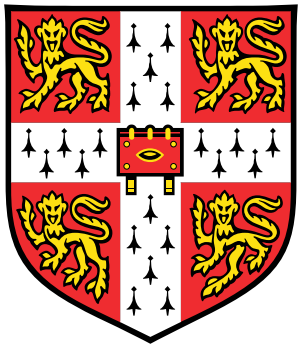
An investigation of the interaction between the immune system GTPase GIMAP6 and an autophagy gene 8 homologue GABARAPL2



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Declaration

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Aamir Mukadam
2014

An investigation of the interaction between the immune system GTPase GIMAP6 and an autophagy gene 8 homologue GABARAPL2

Summary

The guanosine-nucleotide binding family of immunity-associated proteins (GIMAP) is expressed in eukaryotic phyla including a subset of molluscs, vertebrates, and some protists. It is predominantly expressed in the lymphoid organs of mammals and other vertebrates where it plays roles in the homeostasis of the immune system.

My study focuses on human (h) GIMAP6, a cytosolic member of the GIMAP family that is widely expressed across the lymphoid lineages. Studies within our group have uncovered a highly specific interaction between GIMAP6 and hGABARAPL2 (gamma-aminobutyric acid receptor-associated protein-like 2), a mammalian homologue of the autophagy-related protein 8 (Atg8). Using bacterially expressed GABARAPL2 and GIMAP6 I have shown that the interaction between the two proteins is direct. My studies have attempted to gain an understanding of the molecular requirements for this interaction using site-directed mutagenesis and pull-down assays. Mutational analyses, including point mutations within, and truncations of, GIMAP6 and GABARAPL2 have revealed a number of things. Close to its N-terminus GIMAP6 carries a sequence corresponding to a canonical Atg8 interacting motif (AIM), a motif frequently found in proteins that interact with the Atg8 family. My studies indicate, however, that these residues do not play a role in the interaction. Using GTP-agarose I was able to demonstrate that GIMAP6 could bind GDP and GTP and by mutating key residues within its GTP binding domain I could disrupt the interaction of GIMAP6 with GABARAPL2. I have also shown that the C-terminal 10 amino acids of GIMAP6 are necessary for the interaction. Interestingly, variants of GIMAP6 that were unable to bind GTP-agarose were also unable to interact with GABARAPL2, hinting at a crucial role for nucleotide binding in the GIMAP6-GABARAPL2 interaction. Within GABARAPL2, deletion of the N-terminal α-helix resulted in loss of the interaction. A chimeric protein in which the corresponding region in MAP1LC3B, a protein unable to interact with GIMAP6, was replaced by GABARAPL2's N-terminal α-helix reproduced the interaction suggesting that this region is critical for the interaction. Studies in our group have shown that GIMAP6 relocalises to autophagosomes on induction of autophagy. I have shown that variants of GIMAP6 unable to interact with GABARAPL2 fail to display a similar relocalisation.

Finally, recent research has demonstrated that members of the GIMAP family can homo- and hetero-dimerise. I have shown that GIMAP6 can interact with itself and intriguingly, also shows a specific interaction with GIMAP7. Contrary to what was observed for the GIMAP6-GABARAPL2 interaction, truncating the N-terminus of GIMAP6 abrogated the interaction with GIMAP7.

These findings evoke the possibility that the GIMAP GTPases function together in an interacting network.

Acknowledgements

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Abstract

The GTPase of immunity-associated proteins (GIMAP) is expressed in eukaryotic phyla including a subset of molluscs, vertebrates, and some protists. It is predominantly expressed in the lymphoid organs of mammals and other vertebrates where it plays roles in the homeostasis of the immune system.

My study focuses on human (h) GIMAP6, a cytosolic member of the GIMAP family that is widely expressed across the lymphoid lineages. Studies within our group have uncovered a highly specific interaction between GIMAP6 and hGABARAPL2 (gamma-aminobutyric acid receptor-associated protein-like 2), a mammalian homologue of the autophagy-related protein 8 (Atg8). Using bacterially expressed GABARAPL2 and GIMAP6 I have shown that the interaction between the two proteins is direct. My studies have attempted to gain an understanding of the molecular requirements for this interaction using site-directed mutagenesis and pull-down assays. Mutational analyses, including point mutations within, and truncations of, GIMAP6 and GABARAPL2 have revealed a number of things. Close to its N-terminus GIMAP6 carries a sequence corresponding to a canonical Atg8 interacting motif (AIM), a motif frequently found in proteins that interact with the Atg8 family. My studies indicate, however, that these residues do not play a role in the interaction. Using GTP-agarose, I was able to demonstrate that GIMAP6 could bind GDP and GTP and by mutating key residues within its GTP binding domain, I could disrupt the interaction of GIMAP6 with GABARAPL2. I have also shown that the C-terminal 10 amino acids of GIMAP6 are necessary for the interaction. Interestingly, variants of GIMAP6 that were unable to bind GTP-agarose were also unable to interact with GABARAPL2, hinting at a crucial role for nucleotide binding in the GIMAP6-GABARAPL2 interaction. Within GABARAPL2, deletion of the N-terminal α-helix resulted in loss of the interaction. A chimeric protein in which the corresponding region in MAP1LC3B, a protein unable to interact with GIMAP6, was replaced by GABARAPL2's N-terminal α-helix reproduced the interaction suggesting that this region

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Finally, recent research has demonstrated that members of the GIMAP family can homo- and hetero-dimerise. I have shown that GIMAP6 can interact with itself and intriguingly, also shows a specific interaction with GIMAP7. Contrary to what was observed for the GIMAP6-GABARAPL2 interaction, truncating the N-terminus of GIMAP6 abrogated the interaction with GIMAP7. These findings evoke the possibility that the GIMAP GTPases function together in an interacting network.

Nomenclature

ADS	AIM docking site
AIG	avrRpt2-induced gene
AIM	Atg8 interacting motif
ALFY	autophagy-linked FYVE protein
Atg	autophagy-related
Bcl	B-cell lymphoma
BMH	1,6-bis-maleimidohexane
BSA	Bovine serum albumin
CALCOCO2	Calcium-binding and coiled-coil domain-containing protein 2
CLP	common lymphoid progenitor
CMA	chaperone-mediated autophagy
CSB	Complete Sample Buffer
DN	double-negative
DP	double-positive
DSS	disuccinimidyl suberate

E.coli	Escherichia coli
FPLC	fast-protein liquid chromatography
GABARAPL2	gamma-aminobutyric acid receptor-associated protein-like 2
GAD	GTPases activated by dimerization
GAP	GTPase activating protein
GAP	GTPase activating proteins
GATE-16	Golgi-associated ATPase enhancer of 16 kDa
GBP	guanylate-binding protein
GEF	guanine nucleotide exchange factor
GIMAP	GTPase of immunity-associated protein
GNBP	guanine nucleotide binding protein
GST	glutathione-S-transferase
IRG	immunity-related GTPases
LAMP	lysosomal-associated membrane protein
mAb	monoclonal antibody
MAP1LC3	microtubule-associated protein 1 light chain 3
MHC	Major Histocompatibility Complex
mTORC	mammalian target of rapamycin complex
NHS	N-hydroxysuccinimide
NSF	N-ethylmaleimide sensitive factor

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	phosphatidylethanolamine
PI3K	phosphatidylinositol 3-kinase
PPI	protein-protein interaction
PVDF	polyvinylidene fluoride
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SP	single-positive
SRP	signal recognition particle
TBE	Tris-borate-EDTA buffer
TCR	T-cell receptor
TE	$100 \times Tris-EDTA buffer$
TGS	Tris-Glycine-Sodium dodecyl sulphate running buffer
Toc	translocon at the outer-envelope membrane of chloroplasts
ULK	Unc-51-like kinase
Vps	vacuolar protein sorting

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1 Introduction

The subject of this thesis is a family of putative GTPases, encoded by the mammalian GTPase of immunity-associated protein (*GIMAP*) genes, that are predominantly expressed in the immune system. Homologues of this protein family are spread throughout vertebrates including birds, reptiles, fish, with more distant relatives also present in molluscs and higher plants. The mammalian GIMAPs are believed to play a role in the development of lymphocytes and have also been shown to play a role in the regulation of cell death. This thesis focuses on the interaction between GIMAP6 and GABARAPL2 (gamma-aminobutyric acid receptor-associated protein-like 2). Therefore, in this introduction, along with briefly introducing lymphocyte homeostasis and autophagy, I will discuss GTPases and members of the GIMAP family.

1.1 Lymphocyte Homeostasis

The maintenance of homeostasis of the T- and B-lymphocyte populations is crucial for the health of an individual. Lymphocytes are the principal cells of the adaptive immune system; this system evolved in early vertebrates and is capable of immunological memory. Immunological memory enables more efficient recognition of specific pathogens resulting in a stronger and quicker immune response upon re-exposure to the same antigen. During infection, the numbers of lymphocytes increase to fight the invasion and subsequently return to normal levels. The state of homeostasis is perturbed when there are

high numbers of activated lymphocytes in the absence of any infection. To establish lymphocyte homeostasis, lymphocyte development has a number of checkpoints, and the mature lymphocyte population is regulated by appropriate pro- and anti-survival signals. For T-cell development, the checkpoints include T-cell receptor (TCR) mediated-positive and negative selection. Differential TCR interactions with the (peptide)-Major Histocompatibility Complex (MHC) Class I and Class II molecules lead to either pro-survival or pro-apoptotic signals that then result in the activation of cell-survival regulators such as the B-cell lymphoma (Bcl)-2 family proteins. Positive selection provides survival signals to cells that recognise the MHC complex, while negative selection ensures that T-cells displaying an inappropriately high affinity for the MHC complex undergo apoptosis, thus preventing autoimmunity (Starr et al., 2003; Ohashi, 1996). Additionally, low affinity antigen-receptor signals as well as growth factor signals, such as those mediated by IL-7 (reviewed in Mackall et al., 2011), play an important role in the maintenance of appropriate numbers of mature T lymphocytes (Ekert and Vaux, 1997; Kondrack et al., 2003), as does programmed cell death (apoptosis) (Rathmell and Thompson, 2002).

1.2 T-cell development and survival - a brief overview

T-cells begin their development from a common lymphoid progenitor (CLP) in the bone marrow. The progenitor migrates to the thymus and it is in this organ that T-cell development occurs. During the course of T-cell development the CLP undergoes numerous genetically controlled stages that culminate in mature T-cells that express either CD4 or CD8 on their cell surface (Figure 1.2.1). Upon entering the thymus the progenitors express neither CD4 nor CD8 and are called double-negative (DN) thymocytes. They move through the thymus and progress through stages DN1-DN4, as determined by the surface expression of CD25 and CD44 (Figure 1.2.1). At DN3, the thymocytes reach a

critical checkpoint termed β-selection. Rearrangement and expression of the TCRβ allele controls the expression of the pre-TCR, and only thymocytes that express the pre-TCR can progress through to the DN4 stage, the immature single-positive stage ($CD8^+$) and the double-positive (DP) stage ($CD4^+CD8^+$). At the DP stage, further development of the thymocytes depends on the rearrangement of the TCRα locus and expression of a clonotypic αβTCR (Mallick et al., 1993; Dudley et al., 1994). The αβTCR chains have randomly generated specificities and their interactions with the MHC control whether a thymocyte fulfils the requirements to differentiate into single-positive (SP) mature T-cells (Fehling and vonBoehmer, 1997). T-cell development is dependent on cytokine signals notably, IL-7, that affect the expression levels of the pro- and anti-apoptotic members of the Bcl-2 family. The members of this family play an important role in determining the fate of thymocytes as only 5% of all cells leave the thymus to circulate as T-cells in the periphery. The default outcome of cells that do not pass selection is apoptosis, as only cells that receive a TCR-ligation signal of the correct affinity can up-regulate the expression of the anti-apoptotic protein Bcl-2. Cells with too high an affinity or no affinity for the MHC complex undergo apoptosis (Kisielow and von Boehmer, 1995). Hence, at the positive selection stage, only thymocytes that recognise the pMHC complex can survive and, at the negative selection stage those that do are eliminated. How this essential difference in the interpretation of signals of different strength is achieved is still unclear (Fu et al., 2014).

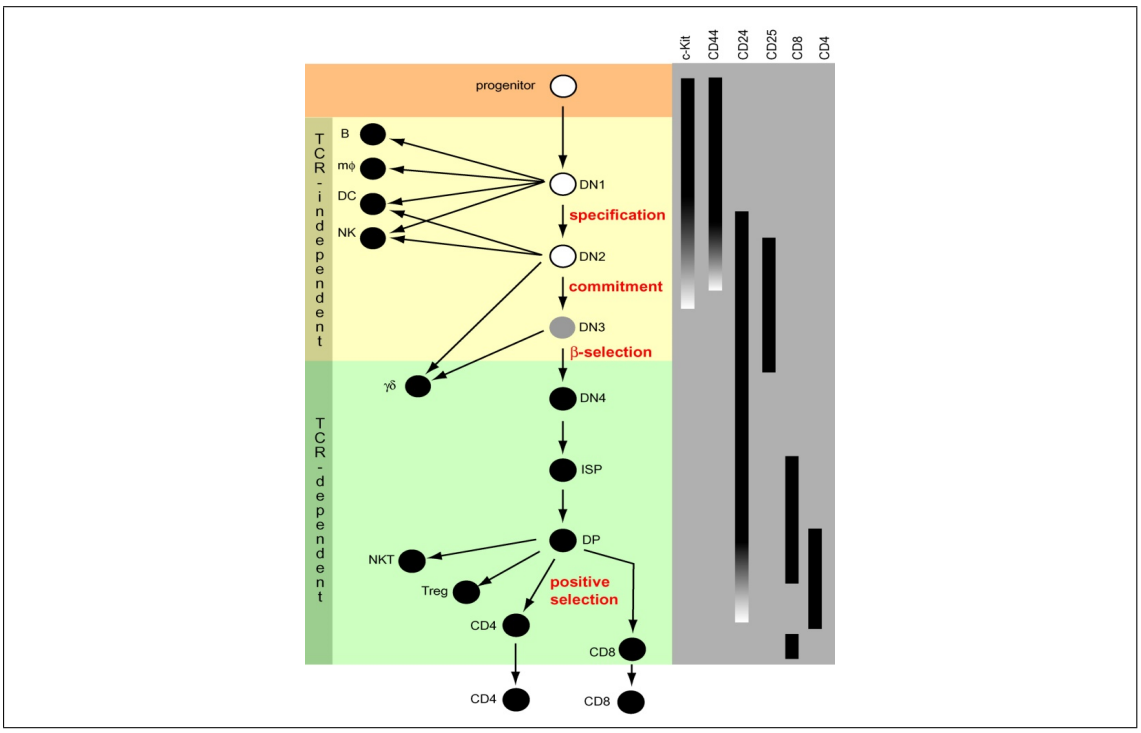

Figure 1.2.1: Outline of T cell development.
Right side-Cell surface markers to identify specific developmental stages. DN: double-negative for both CD4 and CD8. Stages of DN cell differentiation (DN1-DN4) are distinguished by CD44, c-kit and CD25 expression. DP: double-positive for both CD4 and CD8. ISP: immature SP CD8, represents the transition between DN4 and DP stages. Figure taken from Rothenberg and Taghon (2005).

Even T-cells that mature and leave the thymus to circulate in the blood and peripheral lymphoid tissues (lymph nodes, spleen etc.) are not protected from apoptosis. Interaction between the TCR and the MHC is necessary for the continued survival of these cells, with IL-7 and the Bcl-2 family members once again playing crucial roles (Strasser, 2005). T-cells that bear specific antigen receptors are activated when antigens are presented to them during an infection. This triggers the proliferation and differentiation of these T-cells into effector cells and memory cells. Post-infection, the activated T-cells undergo apoptosis, possibly due to the decline in cytokine levels which dampens the expression of anti-apoptotic proteins. Ninety-five percent of activated T-cells die at this stage. The memory cells survive and are now primed to mount a quicker and more effective immune

response should the same pathogen attack the host again (Strasser and Pellegrini, 2004). Recent research has revealed that, in addition to apoptosis, the process of autophagy also has a crucial role in the survival and maintenance of the immune cell population (Pua et al., 2009, 2007; Virgin and Levine, 2009; Levine et al., 2009). Autophagy regulates recycling of proteins and organelles via the lysosome and crucially also plays a role in innate immunity by restricting infections caused by intracellular bacteria, viruses, and parasites (Schmid and Munz, 2007; Munz, 2009; Mostowy and Cossart, 2011; von Muhlinen et al., 2012).

Members of the GIMAP family are implicated in autoimmune diseases (Hornum et al., 2002; Shin et al., 2007; Lee et al., 2013; Lim et al., 2009), and mutations and deletions in some family members, such as GIMAPs 1 and 5, in rodent models, can produce severe lymphopenias (Schulteis et al., 2008; Barnes et al., 2010; Saunders et al., 2010; Elder and Maclaren, 1983). Studies have shown that the expression of proteins within this family can be regulated by TCR signals (Nitta et al., 2006). Members of the GIMAP family have also been shown to be able to bind to pro- and anti- apoptotic proteins of the Bcl-2 family (Nitta et al., 2006; Nitta and Takahama, 2007). Thus, this GTPase family may play important roles in the survival and maintenance of immature and mature lymphocytes. However, little is known about their mechanism of action. As a product of investigations to identify novel protein interactions mediated by the GIMAP family, the Butcher laboratory has recently identified a highly specific interaction between human GIMAP6 and GABARAPL2 , a mammalian homologue of the yeast autophagy protein Atg8 (Pascall et al., 2013). This interaction is the focus of this thesis.

1.3 GTPases

Guanosine triphosphatases (GTPases), a super-family of proteins found in Bacteria, Archaea and Eukaryota, are capable of binding and hydrolysing GTP and have diverse roles.

They are believed to have their origins in translational modifiers and have diversified into distinct forms before the last common ancestor of all modern life forms (Leipe et al., 2002). GTPases function in a variety of fundamental cellular processes, such as cell signalling and motility (heterotrimeric G-proteins, Ras superfamily), membrane trafficking and cytoskeletal dynamics (Rab GTPases, dynamin and tubulins), translation and protein translocation (Ef-Tu and the SRP/SR family) and cell autonomous resistance to infection (interferon inducible GTPases, Mx and p47 GTPases; Martens and Howard, 2006), amongst others.

GTPases share a related structural core that is reflected in sequence similarity and points towards a common evolutionary origin for these proteins (Bourne et al., 1990; Leipe et al., 2002). They contain a ~200-residue guanine nucleotide-binding domain (G domain) that is composed of six-stranded β-sheets surrounded by α-helices (Kjeldgaard et al., 1996). The five polypeptide loops, designated G1 through G5 (Figure 1.3.1), are conserved motifs, with the primary structures of G1, G3 and G4 being the most highly conserved (Dever et al., 1987; Vetter and Wittinghofer, 2001). Based on studies carried out in the Ras superfamily, the loop regions between helix 1 and strand 2 and, between strand 3 and helix 2, are called switch I and switch II, respectively and these are particularly important for the conformational switch mechanism underlying their general mechanism of action. The switch regions primarily bind the γ-phosphate group of GTP with little or no involvement in binding GDP. Switch I contains the G2 motif, and the G3 motif is located at the beginning of switch II (Sprang, 1997).

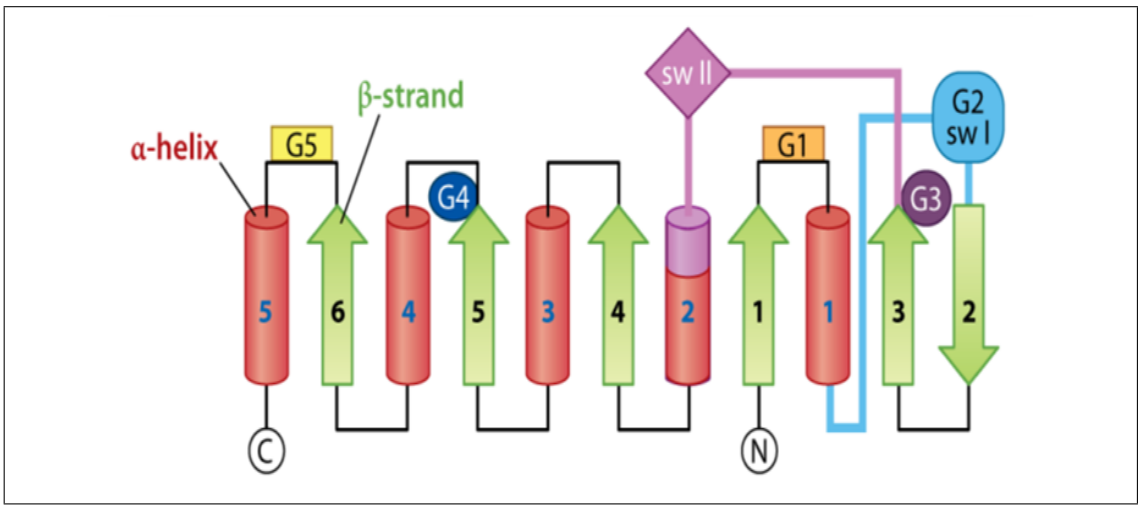

Figure 1.3.1: Topology diagram of the GTPase G domain.
β-strands in green, α-helices in red, and N and C termini as indicated. Conserved motifs are indicated. Figure adapted from Vetter and Wittinghofer (2001).

The G1 motif, also called the diphosphate-binding loop (P-loop), contains the consensus sequence GXXXXGK(S/T) and binds the α- and β-phosphates of the nucleotide molecule (Bourne et al., 1991). The G2 loop contains a conserved threonine residue (XTX) that is required for Mg^{2+} co-ordination (Traut, 1994). The magnesium ion is necessary for the hydrolysis of GTP (Figure 1.3.2). The sequence DXXG, G3, stabilises the interaction between the γ-phosphate of the GTP and Mg^{2+}. The G1 and the G3 motifs are present in several nucleotide-binding proteins; however the specificity of GTPases for guanosine is due to the G4 loop. G4 contains the conserved sequence (N/T)KXD that specifically recognises the guanine ring over adenine. G5, with the consensus sequence (T/G)(C/S)A, strengthens the interaction with GDP/GTP, reviewed in (Sprang, 1997).

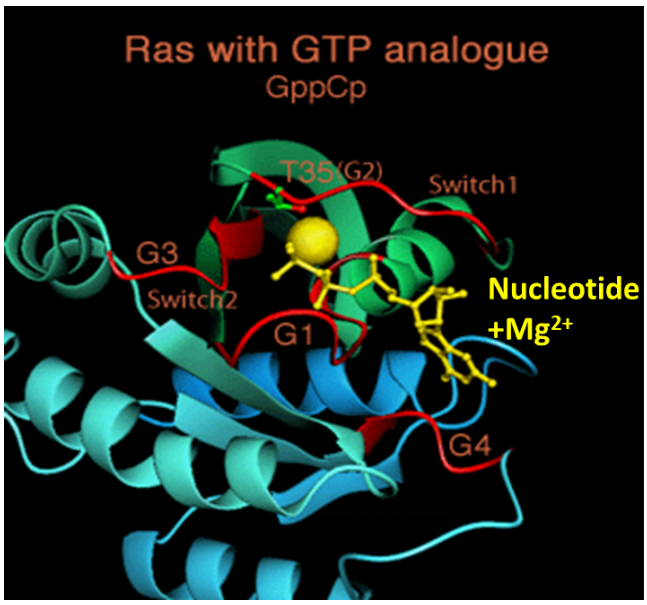

Figure 1.3.2: Schematic representation of the G domain fold.
A schematic diagram of Ras. Switch regions and secondary structure elements are labelled. Mg^{2+} represented by the solid yellow sphere. Figure adapted from McMahon (2004).

1.3.1 Mechanisms of GTPases

Remarkably, the various diverse functions of GTPases are carried out via an almost universally conserved mechanism (Figure 1.3.3; Bourne et al., 1990). The classical mode of action of GTPases, exemplified by the Ras super family of GTPases, is as a molecular switch. GTPases cycle between an 'off/inactive' state when they are bound to GDP and unable to bind downstream effectors, and an "on/active" state when they are bound to GTP and are able to bind downstream effectors (Figure 1.3.3).

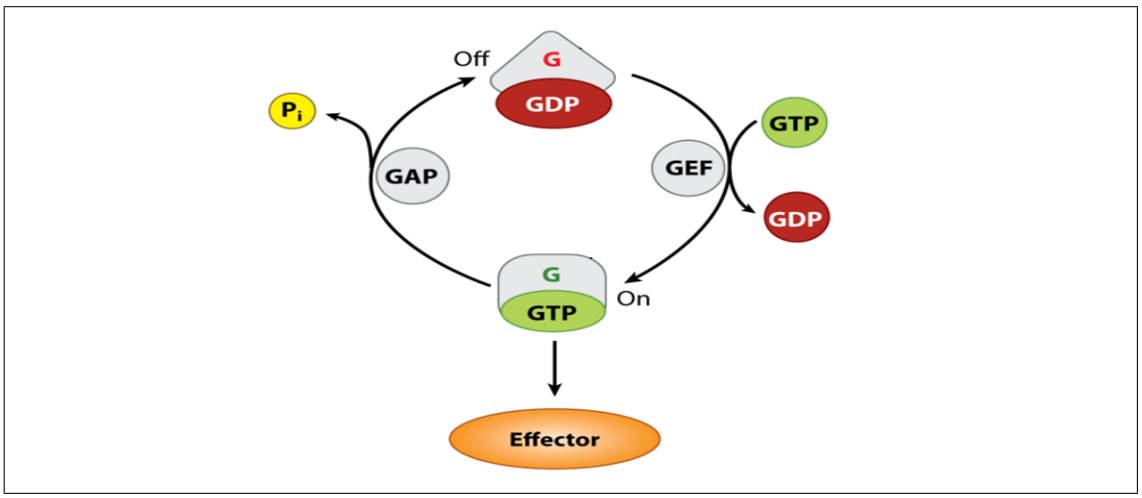

Figure 1.3.3: The functional cycle of Ras-like proteins.
Ras-like proteins are regulated by GEFs and GAPs as depicted. When GTP-bound, GTPases are able to interact with downstream effectors. Figure adapted from Vetter and Wittinghofer (2001).

The molecular switch is well described as a loaded spring mechanism (Vetter and Wittinghofer, 2001). In the on-state, the conserved threonine in switch I (G2 motif) and the glycine residue in switch II (G3 motif) interact with the γ-phosphate of the nucleotide. Stabilised by the γ-phosphate, the switch regions of all small G proteins adopt a remarkably similar, rigid conformation, allowing recruitment and, in some cases, allosteric activation of effector proteins, with one or both switch regions involved in the binding interface. Upon nucleotide hydrolysis and release of inorganic phosphate, the switch-γ-phosphate interactions are disrupted resulting in the switch regions relaxing into a conformation that represents the GDP-bound off state. The presence of divalent magnesium ions is crucial for the interconversion between the functional states as it contributes to catalysis. The ions provide a temporary storage for the electrons taken from the triphosphate and return them back to the diphosphate after bond cleavage and P(i) release (Rudack et al., 2012). Dissociation of GDP and binding of GTP produces a conformational change in the protein resulting in activation of transduction. The release of GDP is intrinsically very slow and the switch between the GDP and GTP bound states is regulated by guanine nucle-

otide exchange factors (GEFs) that catalyse the release of GDP allowing the binding of GTP. The switch regions are unable to interact stably with GDP, with the switch II region disordered in the GDP-bound form and the switch I region interacting only loosely with GDP. The GEFs remodel the switch regions, with the switch II region interacting intimately with the GEFs to induce conformation changes that displace the GDP (Figure 1.3.4). To become 'switched on', GTP must enter the nucleotide-binding site and dissociate the GEF. GTP enters via its GMP moiety and a low-affinity GEF–GTP–small-GTP-binding-protein complex forms and isomerises to bind the β- and γ-phosphate groups, resulting in the dissociation of the GEF (Wang et al., 1997; Kawashima et al., 1996; Cherfils and Chardin, 1999).

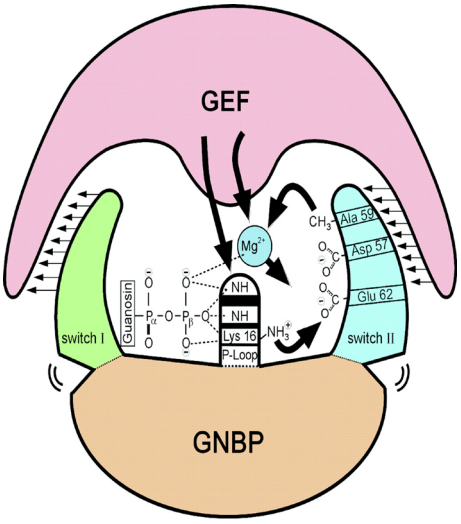

Figure 1.3.4: Mechanistic principles of nucleotide exchange mediated by GEFs. The Mg^{2+} ion is pushed out from the active site either by elements of the GEF or by residues in switch II, which is in turn pulled towards the nucleotide-binding site. The conformation of the P-loop is disturbed by GEF binding, leading to loss of interaction with the phosphates. GNBP-guanine nucleotide binding protein. Figure taken from Vetter and Wittinghofer (2001).

The return to the inactive GDP bound state is regulated by GTP hydrolysis. This is an intrinsically slow reaction and is catalysed by GTPase activating proteins (GAPs), reviewed in (Cherfils and Zeghouf, 2013). The actual GTP hydrolysis reaction, like the

GDP/GTP exchange reaction, is intrinsically very slow within small G proteins as they are inefficient enzymes and GTPase activating protein (GAP) activity is required to catalyse this process (Bos et al., 2007a). An example of GAP-stimulated GTP hydrolysis is the Ras-RasGAP association (Scheffzek et al., 1997). RasGAP increases the GTPase rate of Ras by the following processes: (i) a positively charged arginine residue (the arginine finger) at the binding interface of Ras-GAP inserts into the active site and neutralises the developing negative charge in the transition state of GTP hydrolysis; (ii) A main chain hydrogen bond from the arginine finger of the RasGAP to Gln61 of Ras stabilises this residue (Figure 1.3.5). Gln61, also called the catalytic glutamine, which is located just downstream of the G3 motif, is thought to be important for GTP hydrolysis by positioning a water molecule which in turn acts as nucleophile and attacks the γ-phosphate (Schweins et al., 1995). The arginine finger mechanism is also conserved in other G protein-GAP systems. A pertinent example is the arginine finger within GIMAP7 that enables it to catalyse the GTP hydrolysis reaction in both GIMAP2 and GIMAP7 (Schwefel et al., 2013). Another example is that of the TBC-domain proteins which act as GAPs for Rab GTPases and catalyse the GTPase reaction by inserting both an arginine finger and a catalytic glutamine into the active site. In the case of Rabs, the putative catalytic glutamine within it is involved only in GAP binding and not in GTP hydrolysis (Pan et al., 2006). An exception in the mechanism of GTPase stimulation is the Rap1 GTPase. Here, the GAP provides a catalytic asparagine residue, rather than the canonical arginine residue as the glutamine is missing in the Rap1 active site (Scrima et al., 2008; Daumke et al., 2004). As GAPs play a critical role in the activity of GTPases their activity has a significant impact on GTPase function.

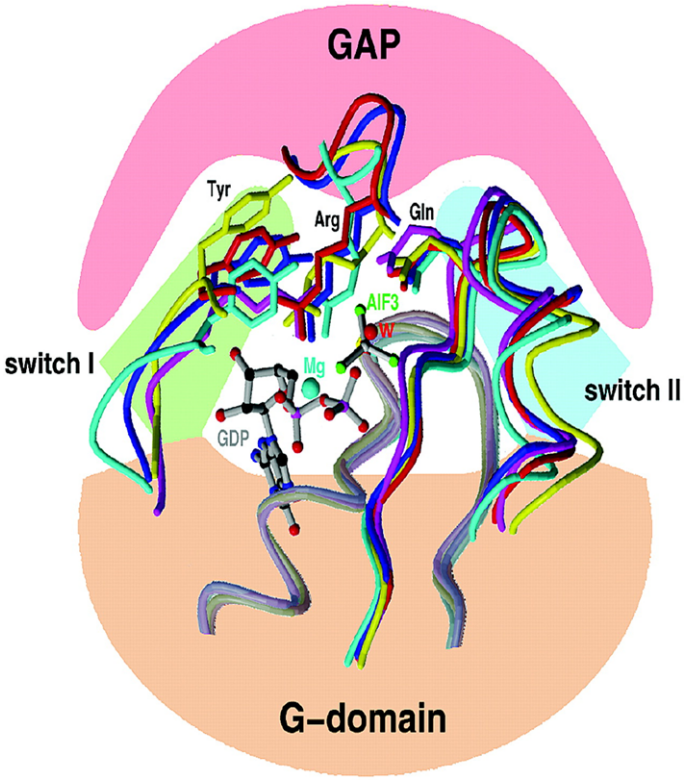

Figure 1.3.5: Conserved mechanism of GTPase-activation for Ras, Rho, Rac and Gα. The switch I and switch II regions of the GTPase are shown in the cartoon representation. Also shown are the GAP loops that provide the arginine fingers. The remainder of the proteins is indicated schematically. Colours refer to protein backbone; Ras-RasGAP is coloured light green, Rho-RhoGAP red, Rac-ExoS cyan and Gα magenta. All structures were solved in the presence of GDP, magnesium and aluminium fluoride, which is a mimic of the transition state during GTP hydrolysis (indicated as AlF3). The catalytic water is indicated as W (in red). The arginine fingers and catalytic glutamines are indicated as Arg and Gln, respectively. Figure taken from Vetter and Wittinghofer (2001).

1.3.2 Subfamilies of GTPases

The mammalian genome encodes hundreds of GTPases that can be split into two main classes based on shared sequence and structural motifs: the TRAFAC (translational factors) and SIMIB1 (signal recognition particle, MinD and BioD) classes (Leipe et al., 2002). GTPases within the TRAFAC class are involved in a myriad of biological functions including translation, signal transduction, motility, and intracellular trafficking. The SIMIB1

class is different from the TRAFAC class in a few significant ways. First, the G1 motif, contains an additional conserved glycine residue, resulting in GxxGxGK[S/T] being the consensus sequence. Second, instead of the conserved threonine within the G2 motif, SIMIBI GTPases contain a conserved aspartate residue. Third, the G4 specificity motif is not as conserved as in the TRAFAC class, and consequently, many SIMIBI members have lost GTP specificity and can exhibit ATPase activity. An example of an NTPase (GTPase) within the SIMIB1 class is the signal recognition particle GTPase (SRP54/Ffh in bacteria) and the signal recognition particle receptor (SR/FtsY in bacteria). These proteins are universally conserved and mediate co-translational targeting of proteins destined for membrane-insertion or secretion (Egea et al., 2005).

GTPases are either monomeric, like those belonging to the Ras super-family, homo- or heterodimeric, like those belonging/related to the septin and dynamin families, or heterotrimeric. The membrane-bound heterotrimeric GTPases, a subfamily of the Ras-like superfamily, are composed of three sub-units: Gα, Gβ and Gγ. These are partners of intracellular G-protein coupled receptors (GPCRs). In the inactive state, GDP is bound to the Gα sub-unit that is closely associated with the G$\beta\gamma$ heterodimer. GPCRs behave like GEFs: binding of the receptors by a ligand catalyses the exchange of GTP for GDP and results in the activation of signal transduction. GTP/GDP exchange occurs at the Gα sub-unit, and results in the dissociation of the heterotrimeric complex into Gα/GTP and G$\beta\gamma$. Both these sub-units are capable of functioning as independent transducers of upstream signals, activating or inactivating various downstream effectors. The intrinsic GTPase activity of the Gα sub-unit results in the re-association of the heterotrimeric complex (Ford et al., 1998; Li et al., 1998; McCudden et al., 2005).

Monomeric GTPases like Ras are homologous to the Gα sub-unit of the heterotrimeric GTPases. However, GTPases are divided into several sub-families, and not all GTPases function as canonical molecular switches (Colicelli, 2004). For instance, the dynamin sub-family of GTPases, a family that is critical for proper endocytosis and vesicle form-

ation, is dependent on self-oligomerisation to carry out its functions (McNiven et al., 2000a,b; Figure 1.3.6).

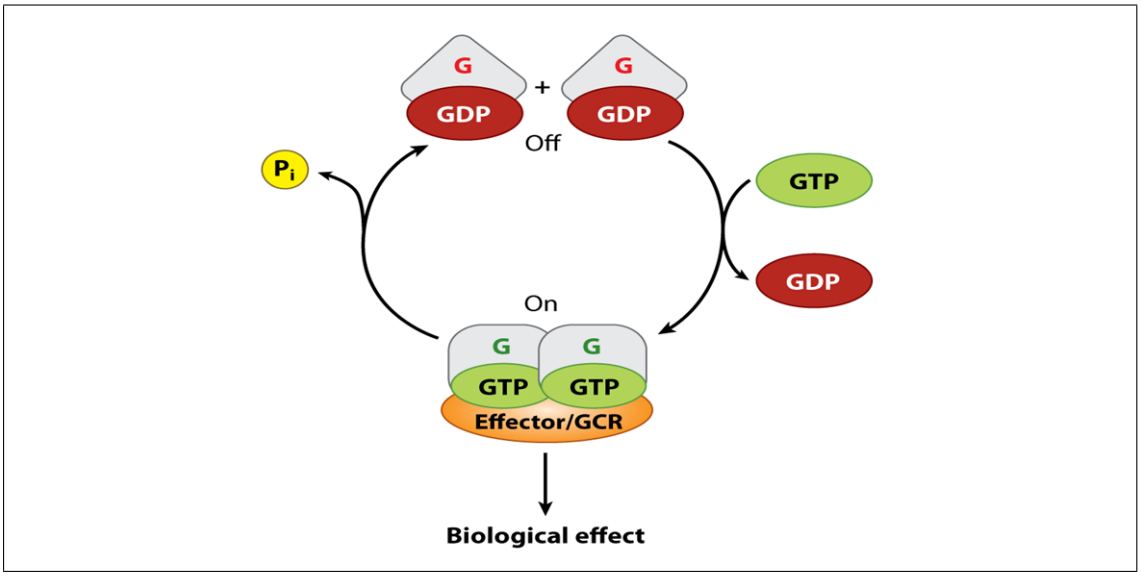

Figure 1.3.6: The functional cycle of GTPases activated by dimerisation. Functional GTPases activated by dimerisation (GAD) do not require a GEF or GAP and can stimulate GTP hydrolysis by homo- or heterodimerisation.

Dynamins use the net motive force generated by the hydrolysis of GTP for the scission of newly formed clathrin-coated vesicles and for the division of organelles (Urrutia et al., 1997). Another group of GTPases that do not function like canonical molecular switches is the septin group. They were first identified in *Saccharomyces cerevisiae* and were shown to participate in cytokinesis (Hartwell, 1971). Septins also play roles in vesicular trafficking (Beise and Trimble, 2011; Trimble, 1999), exocytosis (Beites et al., 1999), and stem cell biology (Hall and Russell, 2012).

Here, I will briefly introduce a few key members of the GTPase family that are of particular interest. These will include the most studied family of GTPases: the Ras-like GTPases; an interesting family of large GTPases, the dynamins; the dynamin-like interferon-inducible GTPases, a family involved in the immunity; the septins, and the paraseptins which include the GIMAP proteins.

1.3.2.1 The Ras-like superfamily

The Ras-like family is the best studied family of GTPases. This superfamily is predominantly eukaryotic, but also includes a few members that are found in prokaryotes. Ras-like GTPases consist of Ras, Rho, Ran, Rab and Arf GTPases. Members of the Ras subfamily generally regulate cell signalling through multiple pathways that include an MAPK (mitogen-activated protein kinase) cascade (Campbell et al., 1998). Rab GTPases, like Arf GTPases (Deretic, 2013), are implicated in the formation, regulation, fusion and movement of vesicles (Burd and Collins, 2004; Hutagalung and Novick, 2011; Hardiman et al., 2012). Interestingly, members of the Ras-like family have also been shown to have fundamental roles in the process of autophagy. Members of the Rab GTPase family in particular are active at various stages of autophagy spanning from autophagosome formation through to autophagosome maturation (reviewed in Ao et al., 2014). The Rho GTPases, through their ability to modulate microtubule dynamics and the actin-myosin cytoskeleton, can regulate a diverse range of cellular functions including cell morphogenesis, migration, division and adhesion (Ridley and Hall, 1992; Chircop, 2014), additionally, a recent study has demonstrated a role for Rho signalling in the regulation of autophagsosome formation (Mleczak et al., 2013). The Ran family is implicated in nucleoplasmic transport (Melchior et al., 1993; Moore and Blobel, 1993; Matchett et al., 2014). Members of the Ras superfamily act like a classic molecular switch (subsection 1.3.1) such that when they are GTP-bound they are able to interact with downstream effectors and regulate critical cellular functions. Despite this mechanistic similarity among the various Ras subfamilies, this family has significant functional diversity.

Ras has been shown to have roles in T- and B-cell development and is also involved in signalling pathways that result in cytokine induction (Genot and Cantrell, 2000; Genot et al., 2000). As mentioned in section 1.2, developing T-cells are required to express a pre-TCR to be able to differentiate and proliferate. Ras has been shown to play an active role at this stage of development, as expression of constitutively active Ras mutants, or the

Ras effector Raf-1, can allow cells to bypass this checkpoint (Iritani et al., 1999; Gärtner et al., 1999; Alberola-Ila et al., 1995). Ras is also stimulated on T-cell activation via the inhibition of protein kinase C (PKC) (Downward et al., 1990). Furthermore, disrupting the activity of Ras has been shown to result in T-cell anergy as it adversely affects IL-2 transcription which is required for the survival of T-cells (Boussiotis et al., 1997). This demonstrates that GTPases are important regulators of B- and T-cell biology.

1.3.2.2 Dynamins

Dynamins were first identified as mechanochemical enzymes that are able to mediate microtubule sliding (Shpetner and Vallee, 1989). Dynamins are larger compared to Ras-like GTPases as they contain additional domains downstream of the G domain that are responsible for self-association, lipid binding, and recruitment of interaction partners. Another aspect that differentiates them from other GTPases is their low nucleotide binding affinities and their tendency to oligomerise, either by themselves or on a lipid template, leading to stimulation of the GTPase reaction. Dynamins play important roles in vesicle scission from the membrane during clathrin-mediated endocytosis and are also involved in a variety of membrane budding processes such as phagocytosis or caveola scission, organelle fusion and fission events, and in regulating the actin cytoskeleton (Taylor et al., 2012; Gu et al., 2010; Sever et al., 2013; Praefcke and McMahon, 2004; Ferguson and De Camilli, 2012). An electron-microscopic study of a bacterial dynamin-like protein has provided an insight into the conformational changes the protein undergoes upon membrane binding and the mechanism by which it causes deformation of the lipid bilayer (Low et al., 2009). Dynamins play roles in mitochondrial fission (Smirnova et al., 2001), which has been shown to regulate oxidative signalling in T-cells (Röth et al., 2014). A recent study has demonstrated that dynamin 2-dependent endocytosis is necessary for lymphocyte egress from both the thymus and lymph nodes by maintaining Sphingosine-1-phosphate (S1P) receptor 1 (S1PR1) signalling (Willinger et al., 2014).

1.3.2.3 Interferon-inducible GTPases

Interferons (IFN) induce numerous gene families that encode effectors and associated regulators, an example of such an induced family of genes is the IFN-inducible GTPase superfamily (MacMicking, 2004, 2012; Martens and Howard, 2006; Taylor et al., 1996). The interferon-inducible GTPase superfamily comprises at least 47 members in humans and mice (Kim et al., 2011). They can be divided into one of four subfamilies on the basis of paralogy and molecular mass: the ~47 kDa immunity-related GTPases (IRG), the ~65–73 kDa guanylate-binding proteins (GBP), the ~72–82 kDa myxoma (MX) resistance proteins, and the ~200–285 kDa very large inducible GTPases (VLIGs) (MacMicking, 2004; Martens and Howard, 2006). Structural analyses of several IFN-inducible GTPases have lead to their being grouped together with members of the dynamin-like family of proteins (Ferguson and De Camilli, 2012; Praefcke and McMahon, 2004).

The IFN-inducible GTPases mediate cell-autonomous immunity, likely by localising to pathogen containing vacuoles, and are expressed in most mammalian cell lineages examined to date (MacMicking, 2012, 2004). One of the pathways employed by this family of proteins in the clearance of pathogens is the autophagic pathway. For example, members of the GBP family help traffic autophagy effector-bound cytosolic proteins to autophagic vacuoles for generating bacteriolytic peptides (Kim et al., 2011). Members of the IRG family also have been shown to confer an autophagic defence against intracellular pathogens (Gutierrez et al., 2004; Singh et al., 2010; Traver et al., 2011). The subcellular localisation of the mouse IRGs is variable, with GTP dependent cycle of IRG-IRG oligomerisation playing a key role in the re-location of IRGs. Unlike the mouse genome which encodes 23 IRGs, the human genome encodes only three putative IRGs, and although only the expression of only the mouse IRGs seems to be regulated by IFN-γ, both the mouse and human IRGM1 GTPases are believed to be required for IFN-γ induced autophagy and anti-mycobacterial activity in macrophages (Virgin and Levine, 2009).

1.3.2.4 Septins

The septin family of GTPases is predominantly more similar to bacterial GTPase families, such as Era, EngA, and TrmE than to the TRAFAC family, indicating that this family may have evolved from bacterial GTPases. Septins are only found in eukaryotes, whereas the other subfamilies (paraseptins and other septin-related GTPases) are wider but more sporadically distributed, extending also to prokaryotic lineages. All members typically contain a divergent version of the guanine recognition motif (G4) at the end of the core strand 5 and an additional helix at the C-terminus of the GTPase domain. Septins were first identified as being involved in the formation of the septum of dividing cells during mitosis and meiosis and have been shown to assemble in rings along the bud neck (Hartwell, 1971). They are able to function as scaffolds that organises the assembly of binding partners. In vertebrates, septins are involved in cell division, cytoskeletal dynamics and secretion (Weirich et al., 2008) and they are able to perform these functions by forming linear, hetero-oligomeric filaments via two distinct interfaces (Sirajuddin et al., 2007). Recent research has demonstrated a key role for septins in innate immunity. By infecting epithelial cell lines with bacteria, Mostowy et al. (2009) demonstrated that septins regulated the process of bacterial entry in epithelial cell lines infected with *Listeria.* Subsequent studies have demonstrated the entrapment of intracytosolic bacteria, *Shigella,* within septin cages and shown an involvement of the septins in autophagic activity (Mostowy et al., 2010). Depletion of members of the septin family adversely affected autophagy. These septin cages with the trapped bacteria are recognised by autophagy cargo receptors, Sequestosome 1 (SQSTM1; otherwise known as p62) and the calcium-binding and coiled-coil domain-containing protein 2 (otherwise known as NDP52 and referred to similarly in this thesis), resulting in the recruitment of microtubule-associated protein 1 light chain 3 (MAP1LC3B) to these cages which are then degraded by autophagy (Mostowy et al., 2011).

The paraseptin family features a diverse group of proteins which includes, amongst oth-

ers, a conserved branch called the AIG1 (avrRpt2-induced gene)-Toc34/Toc159 group (translocon at the outer-envelope membrane of chloroplasts); sequence analysis has indicated that the GIMAP proteins which are the focus of this study belong to the AIG1 branch of the paraseptins (Koenig et al., 2008). AIG1-like proteins are found both in vertebrates and plants where they play roles in anti-pathogen resistance, whereas the Toc group is found only in plants. A defining feature of the AIG1-like proteins is the presence of the G domain along with a functionally undefined, highly conserved sequence termed the conserved box. This sequence, along with the G domain, is present in all members of the GIMAP family. The Toc34/Toc159-like GTPases are mainly found in plants where they function as integral components of the chloroplast protein import machinery (Sveshnikova et al., 2000; Fulgosi and Soll, 2002; Kessler et al., 1994; Kessler and Schnell, 2002). Crystal structures have suggested that Toc34 exists as a dimer with one monomer acting as a GAP for the other (Sun et al., 2002). In Toc33, another member of this family, the dimerisation was required to control the nucleotide-binding state (Oreb et al., 2011; Aronsson and Jarvis, 2011), akin to what is observed in GIMAP2 (Schwefel et al., 2010). The AIG1 subgroup was first identified in *Arabidopsis thaliana* where it was up-regulated when challenged with infection (Reuber and Ausubel, 1996). This subgroup is also found in viruses and bacteria. Intriguingly, AIG1-like proteins are also found in the protozoan parasite *Entamoeba histolytica*, where their expression in pathogenic strains is higher when compared to non-pathogenic ones (Biller et al., 2010), hinting at a potential role for AIG1 proteins in virulence of pathogens as well as in defence mechanisms of the host organism.

1.4 The GIMAP family

GIMAPs or their relatives are expressed in vertebrates, molluscs and higher plants; however, they do not appear to be expressed in unicellular organisms like yeast, or *Caenorhab-*

ditis elegans. GIMAPs are found in certain bacteria, picornaviruses and herpesviruses (Schwefel et al., 2010; Schwefel and Daumke, 2011). The mammalian GIMAP family of proteins is predominantly expressed in the lymphoid organs where they are believed to play roles in lymphocyte development and survival. GIMAPs are putative GTPases that vary between 35-80 kDa, comprising 7-8 genes that are tightly clustered within the genomes of organisms in which they are found, suggesting that they have evolved via gene duplication events. In humans, the GIMAPs are on chromosome 7, and in rats and mice they are on chromosomes 4 and 6, respectively (Figure 1.4.1). The work in this thesis has focussed on human GIMAPs. As mentioned in subsubsection 1.3.2.4, the amino (NH_2)-terminus of all members of the GIMAP family contains the conserved GTP binding AIG1 domain, while the carboxy (COOH)-terminal regions of these proteins display little sequence conservation (Figure 1.4.2). Furthermore, unlike many other GTPases, including members of the Ras super-family, the dynamins, the septins, and indeed several other AIG proteins, mammalian GIMAPs do not have the CaaX motif (C for Cysteine; a for any aliphatic residue; x for any residue) within their C-terminal regions. This motif directs the post-translational addition of a lipid moiety and has critical implications in the localisation and function of the modified protein (Del Villar et al., 1996). A defining feature of the AIG1-like proteins is the presence of the G domain along with a highly conserved sequence termed the conserved box [CYLLSxPGPHVLLLVxQLGRϑTxΨ] where ϑ represents an aromatic amino acid and Ψ represents an acidic amino acid (Schwefel et al., 2013). This sequence, along with the G domain, is present in all members of the GIMAP family. All members contain predicted coiled-coil domains at the carboxy end that may be involved in protein-protein interactions. GIMAP1, 2, 3 and 5 also have transmembrane hydrophobic domains that are likely to influence their intracellular localisation and function.

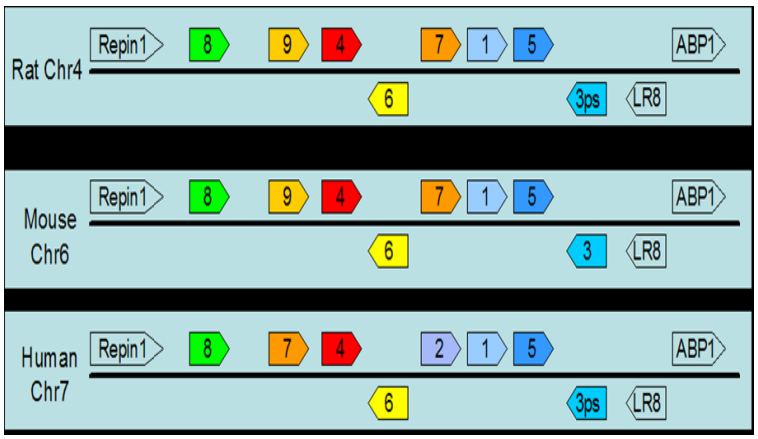

Figure 1.4.1: The organisation of the *GIMAP* gene cluster in the genomes of human, mouse and rat is shown.
The position of each *GIMAP* gene is indicated and the orientation shown with arrows. GIMAP6, which is the research focus of this thesis, is highlighted in yellow. GIMAP3 is a pseudogene (ps) in rats and humans and is denoted accordingly. Genes flanking the GIMAP cluster are shown.

GIMAP1 was the first member of the GIMAP family to be identified. It was identified in a differential screening of a cDNA library made from spleen cells of mice immune to malaria and, those that were not (Krucken et al., 1997). A screen employing the differential display technique to identify genes specifically switched on during positive selection of thymocytes identified more members of the GIMAP family, such as GIMAP5 (Poirier et al., 1999).

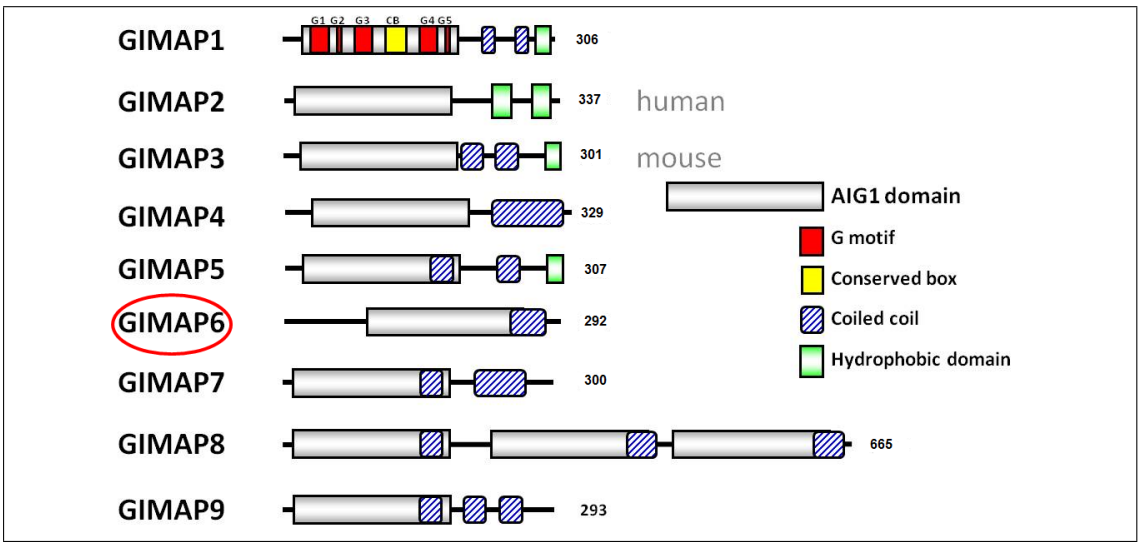

Figure 1.4.2: Schematic representation of GIMAP family members.
In all GIMAP members there is an AIG1 domain (including the 'conserved box') towards the NH_2-terminus of the polypeptides. The COOH-terminus, however, is varied and contains domains which are expected to influence the function and sub-cellular localisation of each GIMAP protein. GIMAP6, which is the focus of this study, is circled in red. Amino acid numbering for human GIMAP proteins.

1.4.1 GIMAPs as G proteins

A Ras-like G1 motif (section 1.3) is highly conserved among the GIMAPs; by contrast, the G3 and G4 motifs are less-canonical. The G3 motif, responsible for the interaction between the γ-phosphate of the GTP and Mg^{2+}, is Ras-like in GIMAPs 4 and 7. In the other GIMAPs, however, the glycine residue of the DXXG motif is replaced by various residues. While a G4 motif is conserved among the GIMAP members, the consensus sequence is altered from (N/T)KXD to T(R/H)KXD. As the G4 motif specifically recognises the guanine ring over adenine, the effect of these changes on the binding and hydrolysing ability of GTP by the GIMAPs is still unknown. To date, few studies have been conducted on examining the GDP/GTP binding and hydrolysing abilities of the GIMAP family. The first evidence for GIMAPs as putative G proteins came from the Calabretta group (Daheron et al., 2001). They demonstrated that HA-tagged mouse GIMAP3 could

bind GTP *in vitro*. Stamm et al. (2002) showed that mGIMAP1 could bind GTP specifically, whereas human (h)GIMAP1 could not. Cambot et al. (2002) showed that hGIMAP4 could bind both GDP and GTP as well as hydrolyse GTP with hGIMAP4, and surprisingly for a GTPase, displayed a greater affinity for GDP than GTP, approximately 12-fold more. However, equilibrium-binding calculations indicated that, in the absence of GTPase activity, hGIMAP4 should still bind GTP, indicating that this molecule could behave like a molecular switch. Crystallographic analyses on GIMAP2 and GIMAP7 carried out by the Daumke group (Schwefel et al., 2010) have shed more light on the GDP/GTP binding properties of the GIMAP family. Their work has demonstrated that hGIMAP2 and hGIMAP7 dimerise in a GTP-dependent manner. They also showed that GIMAP2 exhibited strong GTP binding but no hydrolytic activity. GIMAP7, on the other hand, bound the guanine nucleotides with a lower affinity, but was able to act as a GAP (arginine finger mechanism; section 1.3) and could catalyse the hydrolysis of GTP by both, a homo- and a heterodimerisation (with GIMAP2) mechanism, in a manner similar to what is observed in the septin and dynamin families.

Putting the research on hGIMAPs and their ability to interact with GTP in context, GIMAP2, 4 and 7 have been demonstrated to bind GTP, with only GIMAP4 and GIMAP7 displaying hydrolytic activity. Interestingly, a mouse model with a single amino acid mutation in GIMAP5 within its G1 domain resulted in a phenotype that mimicked the GIMAP5 knockout mouse model (Barnes et al., 2010; Schulteis et al., 2008). These findings point towards interesting, but as yet incompletely characterised roles for the G domain in GIMAP biology. GIMAPs are predominantly expressed in lymphoid organs and members of this family are believed to play important roles in maintaining lymphocyte homeostasis. While some GIMAPs are believed to have pro-apoptotic roles, for example GIMAP4 (Carter et al., 2007; Schnell et al., 2006), others like GIMAP1 and 5 are believed to play a pro-survival role. For example, knocking out the GIMAP1 or the GIMAP5 genes in mice leads to severe T-cell lymphopenia and a reduction in the mature B-cell popula-

tion (Barnes et al., 2010; Nitta et al., 2006; Hellquist et al., 2007; Schulteis et al., 2008; Saunders et al., 2010; Sandal et al., 2003). Findings thus far indicate that the G domain may play an important role in the functioning of the GIMAPs. However, it could also be that the GIMAPs have other, as yet, uncharacterised domain(s) and that these rather than the G domain are necessary for their function(s). Hence, while all GIMAPs may play a role in the functioning of the immune system they could do so via their G domains or independently of it.

As mentioned above, GIMAPs can be divided into two subgroups: one in which the members contain transmembrane domains, and a second group which lack them and are presumed to be, at least in part, cytosolic. The current state of knowledge on the individual GIMAPs will be introduced in the following sections.

1.4.2 GIMAPs with transmembrane domains

1.4.2.1 GIMAP1

Human GIMAP1, 306 amino acids long with a molecular weight of 34 kDa, was the first member of the GIMAP family to be identified. It has the G domain at its NH_2-terminus and a predicted transmembrane domain at its COOH-terminus. During malaria infection gene expression of mGIMAP1 was reported to be increased and was at an elevated level in immune mice both pre- and post-infection, suggesting that GIMAP1 had a role to play in the immunity to malaria (Krucken et al., 1997). However, this increase in GIMAP1 expression was not observed in subsequent studies carried out by the Butcher group (Saunders et al., 2009), either at the mRNA level or at the protein level. In tissue array hybridization experiments, human GIMAP1 was found mainly in spleen and lymph node tissues and to a lesser extent in heart and lung tissues. GIMAP1, on the mRNA level, is up-regulated in T lymphocyte development during the transition from the $CD4^-CD8^-$ DN to the $CD4^+CD8^+$ DP stage in mice and rats (Dion et al., 2005; Nitta et al.,

2006), suggesting a role for GIMAP1 in thymocyte maturation. A quantitative proteomics study showed that GIMAP1 was down-regulated during the differentiation of activated T lymphocytes into T helper 2 cells (Filén et al., 2009).

A conditional GIMAP1 knockout mouse generated by the Butcher group (Saunders et al., 2010) resulted in a phenotype in which extensive lymphopenia was observed, indicating that it plays a vital role in the development and/or survival of T- and B-lymphocytes. The Cre/LoxP method was used to generate the knockout, with the mice expressing the Cre recombinase carrying the hCD2-iCre transgene. hCD2-iCre induces expression of the Cre recombinase in the early stages of development of the T- and B-cells resulting in GIMAP1 being knocked out in these cells only. Studies carried out by the Butcher group indicate that the intracellular localisation of mGIMAP1 is within the Golgi compartment (Wong et al., 2010). A number of Bcl-2 family members can be found at the Golgi (Echeverry et al., 2013) and it could be that the presence of GIMAP1 there is necessary to regulate apoptotic signalling.

1.4.2.2 GIMAP2

GIMAP2 comprises 337 amino acids and has a molecular weight of 38 kDa. It is present in humans but has no orthologue in the rat or mouse. An interesting feature of this protein is that it has two hydrophobic domains at its COOH-terminus. It is predominantly expressed in the spleen, lymph nodes, peripheral blood leucocytes and the thymus (Krucken et al., 2004). The structure of GIMAP2 was recently solved (Schwefel et al., 2010) showing that dimerisation of this protein is dependent on GTP-binding, with GTP-binding leading to the release of constituent amphipatic helix $\alpha 7$ from the G domain. Release of the $\alpha 7$ helix could free it to interact with potential binding partners, for example, other members of the GIMAPs, or indeed members of the Bcl-2 family. The release of this helix on GTP association also leads to the oligomerisation of the protein (Schwefel et al., 2010). Cellular localisation in the Jurkat T cell line of mCherry-tagged GIMAP2, carried out in

the same study, indicated that GIMAP2 predominantly co-localises with lipid droplets (LD) with the hydrophobic domains being required for this localisation. Over-expression of GIMAP2 lead to an increase in the number of lipid droplets. The biological significance of this phenomenon remains unclear. It could be that the GIMAP2 oligomer acts as a scaffold on the LD membrane and plays a role in orchestrating interactions between different proteins.

1.4.2.3 GIMAP3

Mouse GIMAP3 consists of 301 amino acids with a molecular weight of 34 kDa, it is a pseudogene in humans and is absent in rats. It is reported to play a role in the positive selection of CD4 SP and CD8 SP T cells in the thymus (Nitta et al., 2006). Sequence analysis indicates that GIMAP3 is closely related to GIMAP5, and interestingly, recent work by the Takahama group suggests a role for GIMAP3 in the survival of T-cells in the mouse. T-cell numbers were reduced when both GIMAP3 and GIMAP5 were knocked out in mice, compared to knockout of GIMAP5 alone, and a defect in T-cell production was seen in a competitive haematopoietic environment when only GIMAP3 was deleted (Yano et al., 2014). GIMAP3 has a predicted C-terminal helical membrane anchor and has been shown to possess GTP-binding activity (Daheron et al., 2001). Mouse GIMAP3 mRNA is found almost exclusively in spleen and lymph nodes (Nitta et al., 2006). It has been reported to be localised at the mitochondrial outer membrane and via as yet unidentified mechanisms is suggested to be a key regulator of mtDNA segregation (Jokinen et al., 2010; Battersby and Shoubridge, 2001).

1.4.2.4 GIMAP5

GIMAP5 is the most extensively studied member of the entire GIMAP family. It is a 308 amino acid protein with a molecular weight of 35 kDa that is strongly expressed at

the DN stage and at the SP stage of the T-cell lineage. One of the studies that sparked interest in the GIMAP family was the identification of a frameshift mutation in the open reading frame of GIMAP5 in the BioBreeding (BB) rat model of type-1 diabetes. The frameshift led to the last 224 amino acids being replaced with 19 nonsense amino acids resulting in the alteration of the AIG1 domain as well as the loss of the coiled-coiled domain and the transmembrane domain. This mutation resulted in acute T-cell lymphopenia, eventually leading to autoimmunity (MacMurray et al., 2002; Elder and Maclaren, 1983). The $CD8^+$ population of T-cells was severely affected, while a significant decrease was seen in the $CD4^+$ T-cell population. Further studies in the BB rat model demonstrated increased apoptosis of the TCR^{hi} SP thymocytes and peripheral T cells, but no defect in the survival of peripheral B cells (Hernandez-Hoyos et al., 1999). The mutation in the BB rat model results in an alteration in the survival and function of regulatory T cells ($CD8^-4^+25^+$) at the post-thymic level, which could play a central role in the pathogenesis of the disease (Poussier et al., 2005). The importance of GIMAP5 in the immune system was demonstrated in mouse models as well (Barnes et al., 2010; Schulteis et al., 2008). Knocking out GIMAP5 in mice replicated the effects seen in the BB rat model. GIMAP5 deficiency also resulted in blocking natural killer (NK) and NKT cell differentiation and, interestingly, liver failure due to severe apoptosis of the hepatocytes (Schulteis et al., 2008). Another mouse model where the GIMAP5 gene was altered was where an ENU-induced coding mutation (G38C) was introduced in the highly conserved G1 domain of GIMAP5. Changing the single amino acid within the G1 domain replicated the phenotype seen in the knockout mouse model (Barnes et al., 2010), indicating its importance in the functioning of GIMAP5. Taking these findings forward, a recent study demonstrated that $CD4^+$ T cells become Th1/Th17 polarised and cause the development of colitis. The progressive changes in $CD4^+$ T cells are associated with the loss of Forkheadbox group O (Foxo)1, Foxo3, and Foxo4 expression. These findings suggest a novel link between GIMAP5 and Foxo expression thereby providing evidence for a regulatory mechanism

that controls Foxo protein expression and could help maintain immunological tolerance (Aksoylar et al., 2012).

GIMAP5 demonstrates pro-survival properties (Barnes et al., 2010; Saunders et al., 2010; Schulteis et al., 2008), and interestingly, has been shown to interact with both pro- and anti-apoptotic members of the Bcl-2 family (Nitta et al., 2006). GIMAP5 was able to protect Jurkat cells from apoptosis induced by toxins such as okadaic acid as well as apoptosis induced by γ-radiation (Sandal et al., 2003). A recent study, also in Jurkat cells, demonstrated that Notch signalling up-regulated expression of GIMAP5 and protected cells from apoptosis (Chadwick et al., 2010). Notch signalling leads to the activation of the NF-$\varkappa$B signalling pathway that has been shown to inhibit apoptosis. Pandarpurkar et al. 2003 demonstrated that the absence of GIMAP5 in T-cells resulted in T-cell specific cell death and inhibition of caspase 8, but not caspase 9, rescued cells from apoptosis. The observation that caspase 9 inhibition could not rescue GIMAP5 deficient T-cells from apoptosis indicates that GIMAP5 does not function in the canonical intrinsic apoptotic pathway. Recent work, however, has indicated that GIMAP5 plays an important role not just in the survival of T-cells. Deletion of GIMAP5 in mice resulted in an increase in apoptosis of haematopoietic stem cells and progenitor cells leading to a deficient long-term re-population capacity (Chen et al., 2011). GIMAP5 in this study was shown to stabilise the interaction between Mcl-1 and Hsp-70, and this interaction was necessary for the maintenance of mitochondrial integrity in progenitor cells. HSC-70 also interacts with Bcl-2 and Bcl-xL (pro-survival proteins) and GIMAP5 deficiency affects this association. The mechanism of the GIMAP5–HSC-70 interaction is still unknown as is the structural basis of this interaction and provides an exciting avenue for research into GIMAP5. Haematopoiesis was also affected in the mouse models where GIMAP5 was mutated or knocked out (Barnes et al., 2010; Schulteis et al., 2008). In these studies, the abnormalities, excessive haematopoesis amongst others, were limited to the liver. Given that the GIMAP5 knockout mouse does not survive beyond day 14, it is not surprising

that GIMAP5 has a role in the healthy functioning of other organ systems as well.

With regard to the intracellular localisation of GIMAP5, studies by our group have demonstrated, via immunocytohistochemistry and sub-cellular fractionation, that endogenous GIMAP5 is localised predominantly in the lysosome (Wong et al., 2010) and not the mitochondria (Nitta et al., 2006; Pandarpurkar et al., 2003) or the Golgi apparatus as suggested previously (Sandal et al., 2003). Previous studies used transient transfection of tagged GIMAP5 to elucidate its localisation. However, the gross over-expression that results due to transient transfection is likely to have led to physiologically irrelevant findings with regards to this protein's localisation. Additionally, a mass spectrometric analysis of the protein composition of the NK secretory lysosome membrane identified a single GIMAP5 peptide (Casey et al., 2007). Given that GIMAP5 has a role to play in apoptosis, an obvious localisation for it is the mitochondria, as this is where members of the Bcl-2 family are also found and regulate mitochondrial membrane integrity. However, the lysosome is also implicated in apoptosis. Lysosomal membrane permeabilisation can induce apoptosis. It is still unclear whether GIMAP5 has a role to play in maintaining the integrity of the lysosome membrane.

As mentioned above, mutation of GIMAP5 results in autoimmunity. One reason why autoimmune disorders may develop in systems where the GIMAP5 gene is mutated or knocked out is that it may cause an unbalanced survival of T-cells. This was seen by Hernandez-Hoyos et al. (1999) in the BB rat model. They observed that T-cell lymphopenia was due to increased rate of apoptosis of cells that had passed positive selection. Notwithstanding their reduced survival, the SP thymocytes and T-cells are still able to proliferate, rescuing them from death. GIMAP5 deficiency could thus result in the ablation of normal T-cells and a survival advantage for auto-reactive T-cells, eventually leading to a state that is suited for the development of autoimmune disorders. Genetic studies in humans have indicated that a polyadenylation signal polymorphism in GIMAP5 is associated with both systemic lupus erythematosus risk (Hellquist et al., 2007), and an increase

in IA-2 antibodies in patients with type-I diabetes (Shin et al., 2007). An increase in the IA-2 antibodies is a good predictor of onset of type-I diabetes. The detailed mechanisms of the role of GIMAP5 role in lymphocyte signalling pathways are still to be resolved, but work thus far indicates a crucial role for GIMAP5 in the maintenance of a healthy immune system. GIMAP4 and GIMAP5 seem to have opposing roles in terms of survival and it would be interesting to analyse whether they can interact with each. It would also be interesting to analyse an animal model with both GIMAP4 and GIMAP5 knocked out.

1.4.3 GIMAPs without transmembrane domains

1.4.3.1 GIMAP4

A gene expression study identified GIMAP4 as being switched on specifically during positive selection of thymocytes, with its expression being linked to CD3-mediated events (Poirier et al., 1999). It contains 328 amino acids and has a molecular weight of 38 kDa. GIMAP4 has an interesting expression profile: it is undetectable in the early stages of DP development and is only detected in the latter stages (DP TCR^{hi}) as the thymocytes mature to become SP cells (Saunders et al., 2010). GIMAP4 is thus expressed after both β-selection (post DN3) and positive selection. A similar pattern of GIMAP4 expression is seen in the B-cell lineage as well. In the bone marrow, it is detected in the transition between pro-B1 and pre-B1 populations after which it is detected only in the mature B-cell. GIMAP4 is expressed in resting T and B cells, but disappears on lymphocyte activation. Intriguingly, while the protein is undetectable, the mRNA levels remain unchanged pointing to post-transcriptional regulation of GIMAP4 (Cambot et al., 2002). How this regulation takes place has yet to be elucidated. Despite the evidence detailed above regarding the importance of GIMAP4 in lymphocyte development, studies have shown that knocking out the gene has no effect on lymphocyte development (Nitta et al., 2006; Schnell et al., 2006), suggesting functional redundancy with another member of the

GIMAP family. Alternatively, it could mean that GIMAP4 is not required in lymphocyte development.

Although several conserved residues considered to be key for the activity of GTPases, such as Ras, are substituted in GIMAP4, hGIMAP4 binds GDP/GTP and is believed to have an intrinsic GTPase activity (Cambot et al., 2002). hGIMAP4, a cytosolic protein, is also thought to be the only member of the GIMAP family to have an IQ domain that may bind calmodulin (CaM) (Schnell et al., 2006), although the evidence for this is weak and has not been replicated. While GIMAP4 may not be crucial for lymphocyte development, it does play a role in apoptosis (Schnell et al., 2006). Mice deficient in GIMAP4 had peripheral T-cells that displayed delayed apoptosis *in vitro* under serum starvation conditions and other conditions of stress. Following on from this finding the authors determined that while GIMAP4 was not required to initiate apoptosis it was required for the formation and clearance of apoptotic bodies (Schnell et al., 2006). In addition, over-expressing GIMAP4 in foetal thymus organ culture (FTOC) accelerated apoptosis of DP thymocytes (Nitta et al., 2006). Further evidence of GIMAP4 being important for apoptosis came from the identification of a hypomorphic variant of GIMAP4 in the Brown Norway (BN) rat by the Butcher group. An AT insertion in this rat allele truncates the protein towards the C-terminus and results in a delayed apoptosis phenotype (Carter et al., 2007), similar to that of the mouse knockout. GIMAP4 has been shown to interact with the pro-apoptotic protein Bax (Nitta et al., 2006) by co-immunoprecipitation, thus providing a mechanism for its ability to modulate apoptosis. GIMAP4 expression levels respond to TCR signals at the DP thymocyte stage and increase (Nitta et al., 2006), and it could be that GIMAP4 plays an important role in negative selection. In fact, GIMAP4 expression levels were significantly higher in apoptotic/negatively selected thymocytes than in non-apoptotic cells (Poirier et al., 1999). Interestingly, GIMAP4 has conserved sites of phosphorylation and there is evidence that these may play a role in its ability to regulate apoptosis. GIMAP4 is a target of PKC phosphorylation and phosphorylation was at a higher level in dead

cells than in live cells (Schnell et al., 2006). GIMAP4 is believed to act downstream of caspase-3 activation and, interestingly, PKC activation is dependent on caspase-3 (Zhang et al., 2005). Further investigations to determine how GIMAP4 carries out its functions are necessary to pinpoint its exact role in lymphocyte development and survival. Discovering interacting partners for GIMAP4 would aid in characterising its function.

1.4.3.2 GIMAP6

GIMAP6 is a cytosolic protein on which little work has been published that has 292 amino acids and a molecular weight of 33 kDa. It is widely expressed across the lymphoid lineages and in mice has a higher expression level in B-cells when compared with the other GIMAPs. In thymocytes, GIMAP6 mRNA is highly expressed at the DP stage (Dion et al., 2005). GIMAP6 mRNA levels in the diabetes-resistant rat, in which GIMAP5 is absent, were reduced in the spleen and mesenteric lymph nodes but not in the thymus when compared to rats that did not have GIMAP5 knocked out in this system (Rutledge et al., 2009). GIMAP expression (mRNA) was reduced in non-small cell lung cancer, with GIMAP6 expression being significantly reduced in the tumour cells (Shiao et al., 2008). The function of GIMAP6 is still uncharacterised, however, our group has recently demonstrated a highly specific interaction with a mammalian homologue of the yeast autophagy gene Atg8, GABARAPL2 (Pascall et al., 2013). This interaction is studied in this thesis.

1.4.3.3 GIMAP7

Human GIMAP7 consists of 300 amino acids with a molecular weight of 34.5 kDa. As in GIMAP4, approximately the last 100 amino acids are predicted to form a coiled-coil region. In mice, its mRNA is detectable in thymus, spleen, lymph node and lung tissue, and it is expressed in B and T cells (Nitta et al., 2006). In rats, it is most strongly expressed

in peripheral T cells. In mice and rats, its expression in thymocytes is low and unchanged over all developmental stages (Dion et al., 2005; Nitta et al., 2006). GIMAP7, along with GIMAP2, is amongst the 15 most downregulated genes in human anaplastic large cell lymphomas (Eckerle et al., 2009). GIMAP7 is highly expressed in peripheral T-cells and displays a similar pattern of expression to GIMAP4 (Dion et al., 2005). It is expressed in the spleen, lymph nodes, and thymus but is not highly expressed in B-cells.

A recent structural analysis of GIMAP7 revealed that it can act as a GAP with the catalytic arginine finger in the GIMAP7 homodimer and GIMAP7-GIMAP2 heterodimer serving a dual function by promoting self-association and stimulating GTP hydrolysis (Schwefel et al., 2013). Like GIMAP2, GIMAP7 was also observed to localise to lipid droplets in Jurkat T cells. What implications this interaction has for T-cell biology needs elucidating, and identifying further interacting partners may help reveal its function and mechanism of action.

1.4.3.4 GIMAP8

GIMAP8 has three putative GTP binding domains making it unlike any other member of the GIMAP family or indeed any other GTP binding protein. It is unknown whether any or all of the G domains within this protein are active and GIMAP8 is as yet functionally uncharacterised. GIMAP8 was identified as a single protein rather than three different proteins by the Butcher group (Dion et al., 2005). It was first discovered in mice and contains 688 amino acids with a molecular weight of 77 kDa. It was reported to have anti-apoptotic properties (Krücken et al., 2005) although this finding has not been replicable by the Butcher group. GIMAP8 expression was reduced in the GIMAP5 deficient rat, however this was not reflected in the mRNA levels, suggesting the occurrence of post-transcriptional regulation (Dion et al., 2005). While GIMAP8 is predicted to be cytosolic, work in the Butcher group (Hepburn L. PhD Thesis, University of Cambridge) has indicated that a proportion of it could be loosely bound to a membrane. mGIMAP8

is expressed in the lymph nodes, and to a lesser extent in the spleen, thymus and lung. Interestingly, while GIMAP8 expression was reduced in non-small cell lung cancers, it was highly expressed in the surrounding non-tumour tissues (Shiao et al., 2008). Why this should be is unknown. Additionally, GIMAP8 knockout mice are viable (Pascall/Webb unpublished).

1.5 Autophagy

As this thesis focusses on the interaction between the human GTPase GIMAP6, and, GABARAPL2, a mammalian homologue of the yeast autophagy protein Atg8, I will briefly introduce the autophagic pathway.

Autophagy is a conserved cellular process that mediates turnover of cellular constituents in the lysosomes. Depending on the type of autophagic cargo being delivered to the lysosome, autophagy can be divided into three different pathways: macroautophagy (hereafter referred to as autophagy and the type of autophagy referred to in this thesis), microautophagy and chaperone-mediated autophagy. Macro-autophagy delivers cytoplasmic cargo to the lysosome via a double membrane-bound vesicle, called the autophagosome, that fuses with the lysosome to form an autolysosome. The process in which cytosolic components are directly taken up by the lysosome itself through the invagination of the lysosomal membrane is termed micro-autophagy. Both macro-and micro-autophagy are able to engulf large structures through both selective and non-selective mechanisms. However, in chaperone-mediated autophagy (CMA), targeted proteins are degraded by being translocated across the lysosomal membrane in a complex with chaperone proteins (e.g. Hsp-70) that are recognised by the lysosomal membrane receptor lysosomal-associated membrane protein 2A (LAMP-2A).

Autophagy was initially characterised as a survival mechanism induced in response to nutrient deprivation (starvation), leading to the production of metabolites required to syn-

thesise essential molecules and ATP that is required for cell survival (Lum et al., 2005b,a; Kuma et al., 2004). A major player in nutrient sensing is the protein kinase mammalian target of rapamycin complex 1 (mTORC1), which is a key regulator of cell growth and autophagy. During favourable growth conditions, mTORC1 is active and acts to inhibit autophagy, whereas in periods of stress (nutrient deprivation), mTORC1 is inactive and autophagy is initiated, likely due to the release of mTOR-mediated inhibitory phosphorylation of Unc-51-like kinase (ULK)-1/2 in mammalian cells (Ganley et al., 2009). Autophagy is tightly regulated as it participates in diverse processes including stress management, ageing, development and the immune response, amongst others (Mizushima and Levine, 2010; Vellai et al., 2009; Levine and Deretic, 2007).

Autophagy can be non-selective or selective. Starvation-induced autophagy involves random sequestration of cytoplasmic components within double-membrane vesicles termed autophagosomes (Kopitz et al., 1990). Selective autophagy performs critical quality control functions that involve selective sequestration of substrates that include soluble proteins, protein aggregates, damaged organelles (mitochondria, peroxisomes, lipid droplets, amongst others), and invasive microbes (Yang and Klionsky, 2010).

1.5.1 Molecular machinery required for the formation of autophagosomes

The identification of about 30 autophagy-related (Atg) genes, using yeast genetic screens, has helped greatly in elucidating the core molecular mechanisms of autophagy (Thumm et al., 1994; Harding et al., 1995; Tsukada and Ohsumi, 1993). Several Atg genes are conserved from yeast to man, an exception being the mammalian-specific Atg101 (Hosokawa et al., 2009), and encode proteins that are required for autophagy. Studies in yeast have demonstrated that the Atg proteins are predominantly localised to cytosolic puncta termed the phagophore assembly site (PAS) and the assembly of the Atg proteins at this site

occurs in a hierarchical manner (Suzuki et al., 2001). However, in mammalian cells, the co-localisation of Atg proteins is observed at multiple sites, instead of a single PAS (Young et al., 2006; Yamada et al., 2005; Mizushima et al., 2001). This phagophore expands to engulf intracellular cargo, and sequesters it in the double-membraned autophagosome (Figure 1.5.1). This then fuses with the lysosome, promoting the degradation of autophagosomal contents by lysosomal acid proteases (Glick et al., 2010). The PAS forms constitutively, and under basal conditions a key component that marks this site is Atg11. However, upon the induction of autophagy, Atg11 is replaced by the Atg17-Atg31-Atg29 complex that assembles at the PAS along with Atg1 and Atg13 (Mao et al., 2013; Cheong and Klionsky, 2008). Atg9 plays an important role in directing membrane to the PAS for autophagosome formation by shuttling between the PAS and peripheral sites. Subsequently, the Atg12–Atg5-Atg16 complex is recruited to the PAS where it acts in a manner akin to that of an E3 ligase to facilitate the formation of lipidated Atg8 (subsection 4.1.3) (Feng et al., 2014).

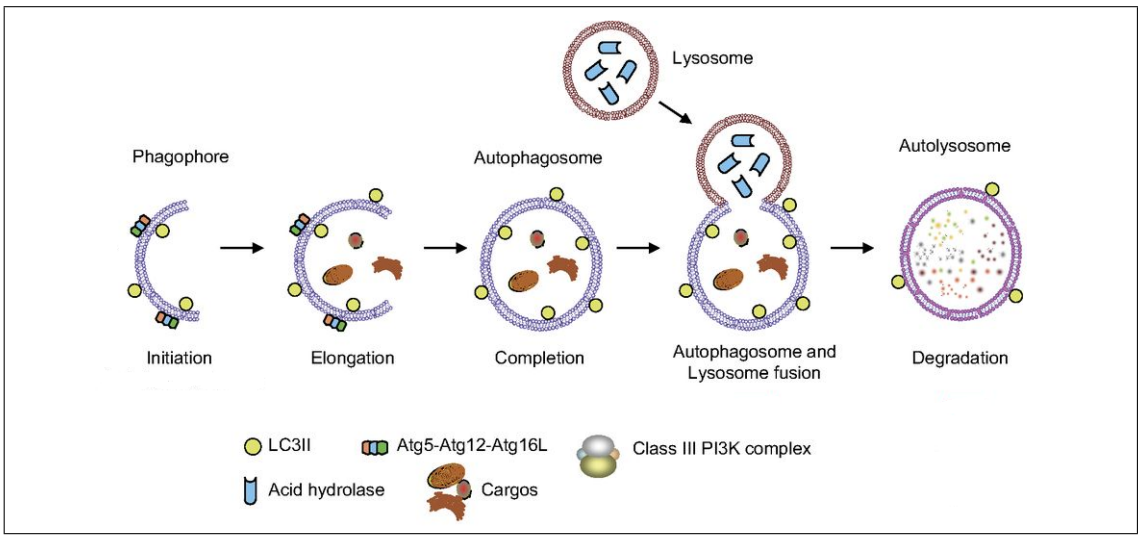

Figure 1.5.1: Process of autophagic degradation.
A schematic representation of the process of autophagy. The ULK and the PI3K complexes are required for the phagophore formation. The Atg-Atg12-Atg16L complex and MAP1LC3B-II sequester the cytosolic cargo and promote phagophore elongation and autophagosome formation. The lysosome fuses with the autophagosome forming the autolysosome and this is where degradation of the cargo occurs. Figure adapted from Nakahira and Choi (2013).

Briefly, the machinery for autophagosome formation is made up of the following functional subgroups: 1) the Atg1/ULK complex (Atg1, Atg11, Atg13, Atg17, Atg29 and Atg31) that regulates the induction of autophagosome formation; 2) the Atg9 and its cycling system (Atg2, Atg9 and Atg18) that plays a role in delivering membrane to the expanding phagophore after the assembly of the Atg1 complex at the PAS; 3) the autophagy-specific class III phosphatidylinositol 3-kinase (PI3K)/vacuolar protein sorting (vps) 34 complex (Vps34, Vps15, Vps30/Atg6, and Atg14) that acts at the stage of vesicle nucleation, where it is involved in the recruitment of phosphatidylinositol-3-phosphate binding proteins to the PAS; 4) two ubiquitin-like (Ubl) conjugation systems: the Atg12 (Atg5, Atg7, Atg10, Atg12 and Atg16) and Atg8 (Atg3, Atg4, Atg7 and Atg8) conjugation systems that play key roles in vesicle expansion and maturation (Suzuki et al., 2001; Xie et al., 2008; Mizushima, 2007). As GIMAP6 interacts with a mammalian homologue of the yeast Atg8, I shall focus on the ubiquitin-like conjugation systems in this thesis.

In yeast, there are two Ubl protein conjugation systems that participate in autophagy. These include two Ubl proteins, Atg8 and Atg12, that are used to generate the conjugation products Atg8-PE and Atg12-Atg5, respectively. These conjugation systems participate in phagophore biology, although their precise functions are not yet fully understood. The Atg12-Atg5 conjugate, along with a third component, Atg16, is thought to act like an E3 ligase by facilitating the conjugation of Atg8 to PE. The amount of Atg8 can regulate the size of autophagosomes (Young et al., 2006) and it also has functions in cargo binding during selective autophagy (Velikkakath et al., 2012). Although the Ubl protein conjugation systems are highly conserved from yeast to mammals (Ohsumi, 2014), one primary difference is the existence of multiple homologues of yeast Atg8. These are divided into two subfamilies, the MAP1LC3 subfamily and the GABARAP subfamily. Refer Chapter 4 for further details on the MAP1LC3 and GABARAP subfamilies.

Atg12 is a unique ubiquitin-like molecule, that is 2.5 times larger than ubiquitin, and is synthesised as an active form without the processing of its C-terminus. The C-terminal glycine of Atg12 is first activated by the E1 enzyme Atg7, and is then transferred to an E2 enzyme, Atg10, before finally forming a conjugate with Atg5 (Mizushima et al., 1998). This Atg12-Atg5 conjugate is essential for autophagy, although further work needs to be done to reveal its precise role(s) in this process. Subsequent to the identification of the Atg12 conjugation system, another conjugation system, the Atg8 conjugation system, was discovered among the Atg proteins. Atg8 is a small hydrophilic protein whose expression is up-regulated upon starvation (Kirisako et al., 1999). Starvation also causes a re-location of Atg8 to the autophagosome (Kirisako et al., 2000), and therefore Atg8 is a useful marker of the autophagic activity. Biochemical studies revealed a tightly membrane-associated form of Atg8 (Kirisako et al., 1999), and additional work illustrated the role of a unique ubiqutination-like modification system (Ichimura et al., 2000). Atg7 is unique in that it is able to activate two ubiquitin-like molecules, Atg12 and Atg8, and transfers them to different E2 enzymes. Given that they share a common E1 enzyme, it follows that

these two conjugation systems function in a closely related manner. Indeed, Atg12-Atg5 functions as an E3-like enzyme to enhance the Atg8 lipidation reaction (Hanada et al., 2007; Sakoh-Nakatogawa et al., 2013).

1.5.2 Autophagy and Immunity - A brief overview

Recent analyses have revealed complex connections between autophagy and immune signalling. Given that autophagy is believed to have evolved as a stress response to enable survival in harsh conditions, it seems likely that it would diversify to counter stress imposed by invasive pathogens and inflammation, amongst other sources of stress. Indeed, several studies have demonstrated key roles for autophagy in innate immunity (reviewed in Deretic, 2012).

Autophagy has also been shown to play key roles in adaptive immunity by being involved in the regulation of development and homeostasis of the immune system, and in the regulation of antigen presentation. Autophagy is involved in both cross-delivery of antigens from intracellular pathogens thereby contributing to the development of an efficient immune response, and in the delivery of endogenous antigens for MHC class II presentation to $CD4^+$ T cells (Cuervo and Macian, 2014; Paludan et al., 2005; Deretic, 2013; Schmid and Munz, 2007). Knockout studies of autophagy genes in specific lymphocyte populations in mice have revealed roles for autophagy in the maintenance of normal numbers of B1a B cells (Miller et al., 2008), $CD4^+$ T cells, $CD8^+$ T cells (Pua et al., 2007) and foetal haematopoietic stem cells (Liu et al., 2010). Deletion of Beclin-1 in $CD4^+$ T-cells inhibits autophagy and causes an accumulation of apoptotic proteins, resulting in cell death (Kovacs et al., 2011). Deletion of Atg5, a gene essential for autophagy, resulted in reduced numbers of both thymocytes and mature T-cells (Pua et al., 2007, 2009). Interestingly, the lymphopenic T cell phenotypes of GIMAP5-deficient rats and mice is also seen when Atg5 and Atg7 have been ablated conditionally in the T cell lineage. In both cases, while intrathymic T cell development is relatively normal, the peripheral T cell population is

severely depleted and there is a marked absence of resting, naïve T cells. Genetic disruption of Atg5 in thymic epithelial cells also leads to altered selection of certain MHC class II restricted T-cell specificities and autoimmunity (Nedjic et al., 2008). Conversely, induction of autophagy in T-cells has also been shown to induce apoptosis (Biard-Piechaczyk et al., 2006). These two divergent phenomena have yet to be completely reconciled.

2 Materials and Methods

All results shown in this thesis are representative of at least 2 independent experiments.

2.1 Molecular biology techniques

2.1.1 Polymerase chain reaction

To keep cross-contamination to a minimum, all polymerase chain reactions (PCR) were performed using aerosol-resistant and nuclease-free pipette tips, and stocks of autoclaved ddH2O kept specifically for PCR. Amplification of the DNA sequences was performed in a PTC-100 Peltier thermal cycler (MJ Research Inc, USA). Amplification of cDNA sequences for the purpose of cloning was performed using Accuzyme™ DNA polymerase (Bioline). Each reaction was performed in a volume of 50 μL and was performed using 2.5 U enzyme, 50pmol primers (purchased from Invitrogen Life Technologies), 400 μM dNTPs, 5 μL of the supplied 10x PCR buffer, 30 ng of plasmid DNA template and 5% (v/v) DMSO to facilitate melting of GC-rich regions. A negative control containing no template DNA was included in every experiment. Following initial denaturation of template DNA for 3 min at 95 ºC, the reactions were subjected to 35 cycles with the following protocol: 1. Denaturation for 30 s at 95 ºC; 2. Annealing for 30 s at a temperature 60 ºC; 3. Extension for 4 min at 72 ºC. The final cycle had an extension at 72 ºC for 10 min. On completion of the programmed cycles, the reaction was maintained at 4 ºC by the thermal

cycler. Samples were subsequently stored at -20 ºC. Products generated were analysed by agarose gel electrophoresis.

Table 2.1.1: Reagents: Buffers and Solutions.

Reagent	Description
1x Phosphate Buffered Saline (PBS)	8 g NaCl, 0.2 g KCl, 1.44 Na_2HPO_4, 0.24 g KH_2PO_4. pH 7.4. Appropriate volume of dH_2O added to make up to 1 L.
10x TBE (TBE)	108 g Tris base, 55 g boric acid, 40 mL 0.5 M EDTA (pH 8.0). Appropriate volume of dH_2O added to make up to 1 L.
Luria-Bertani broth (LB)	Recipe made up to 1 L by adding appropriate volume of dH_2O. Tryptone 10 g, NaCl 10 g, Yeast extract 5 g.
LB-agar	Recipe made up to 1 L by adding appropriate volume of dH_2O. Tryptone 10 g, NaCl 10 g, Yeast extract 5 g, 15 g Agar.
100 × Tris-EDTA buffer (TE)	39.4 g Tris-HCL 9.03 g EDTA. Appropriate volume of dH_2O added to make up to 250 mL (pH adjusted to 8 using NaOH).
Agarose loading buffer	0.25% (w/v) bromophenol blue, 30% (v/v) glycerol
1 × Complete Sample Buffer (CSB)	80 mM Tris pH6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol or 200 mM dithiothreitol
2 × Complete Sample Buffer (CSB)	160 mM Tris pH6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol or 400 mM dithiothreitol, 0.008% (w/v) bromophenol blue
10 × Tris-Glycine-Sodium dodecyl sulphate running buffer (TGS)	0.25 M Tris, 1.92M glycine, 1.0% SDS, use at 1 × dilution
Transfer buffer	39 mM glycine, 48 mM Tris, 0.0375% SDS, 20% methanol
Blocking buffer (Western)	5% (w/v) Marvel milk powder in 1 × PBS containing 0.1% (v/v) Tween 20
Wash buffer (Western)	0.1% (v/v) Tween 20 in 1 × PBS
E. coli lysis buffer	5 mM EDTA (pH 8.0), 1% (v/v) Triton X-100, 5 mM $MgCl_2$
Triton X-100 lysis buffer	10 mM HEPES, 150 mM NaCl, 1% (v/v) Triton X-100, pH 7.5
GTP lysis buffer	50 mM Tris, 150 mM NaCl, 10 mM $MgCl_2$, and 0.1% Triton X-100, pH 7.4
NP40 lysis buffer	150 mM NaCl, 1% NP40, 50 mM Tris, pH 8.0
SEC buffer	20 mM Tris-HCl pH 7.5, 150 mM

2.1.1.1 Site-directed mutagenesis

Site-directed mutagenesis was performed employing a PCR-based strategy with the Accuzyme™ DNA polymerase (Bioline) with the primers listed in Table 2.1.2. N- and C-terminally truncated fragments were generated using oligonucleotides located at the desired ends of the fragments. Where necessary, internal site-specific mutations were introduced by generating two overlapping PCR products incorporating the desired mutation within the overlapping region. These were then joined in a second PCR reaction spanning the whole desired fragment (primers JP539 and JP541; Table 2.1.2). The inserts of all PCR-derived plasmids were sequenced completely by Cogenics to ensure that only the desired mutations, and nothing else, had been introduced.

Table 2.1.2: List of primers.

F: forward primer, R: reverse primer. Forward primers incorporated the *Eco*R1 restriction site (underlined in red), while reverse primers incorporated the *Xho*I restriction site (underlined in black).

Primer name	Primer sequence (5´- 3´)
JP539 (f)	GCGGAATTCTGATGGAGGAAGAAGAAGAATATG
JP541 (r)	GCGCTCGAGAAGGTCAGCCTTCCCCAGC
GIMAP6 $_{Y6A}$ (f)	GCGGAATTCTGATGGAGGAAGAAGAAGCTGAACAAATTCC
GIMAP6 $_{I9A}$ (f)	GCGGAATTCTGATGGAGGAAGAAGAATATGAACAAGCTCCCCAG
GIMAP6 $_{G50D}$ (f)	GAAAACAGACAGTGGGAAG
GIMAP6 $_{G50D}$ (r)	CTTCCCACTGTCTGTTTTC
GIMAP6 $_{S54N}$ (f)	GGGAAGAATGCAACAGG
GIMAP6 $_{S54N}$ (r)	CCTGTTGCATTCTTCCC
GIMAP6 $_{T76A}$ (f)	CAGCACCAGACCCGTGGCCAAGACCTCCCAGAG
GIMAP6 $_{T76A}$ (r)	CTCTGGGAGGTCTTGGCCACGGGTCTGGTGCTG
GIMAP6 $_{D95A}$ (f)	GAGCTTGAGGTGATTGCCACACCCAAC
GIMAP6 $_{D95A}$ (r)	GTTGGGTGTGGCAATCACCTCAAGCTC
GIMAP6 $_{N98A}$ (f)	GTGATTGACACACCCGCCATTCTGTCCC
GIMAP6 $_{N98A}$ (r)	GGGACAGAATGGCGGGTGTGTCAATCAC
GIMAP6 $_{R134A}$ (f)	GTGACACAACTGGGCGCCTTCACGGATGAGGAT
GIMAP6 $_{R134A}$ (r)	ATCCTCATCCGTGAAGGCGCCCAGTTGTGTCAC
GIMAP6 $_{D167A}$ (f)	TTCACCCGGAAGGAAGCCCTGGCTGGCG
GIMAP6 $_{D167A}$ (r)	CGCCAGCCAGGGCTTCCTTCCGGGTGAA
GIMAP6 $_{N201A}$ (f)	GCGCCATTGCGGCTTCAACGCCAGGGCACAG
GIMAP6 $_{N201A}$ (r)	CTGTGCCCTGGCGTTGAAGCCGCAATGGCGC
GIMAP6 $_{37-292}$ (f)	GCGGAATTCTGACCCCAAGGAGACTGAGGCTC
GIMAP6 $_{1-243}$ (r)	GCGCTCGAGAAAGTTTTGCTGGGTATATTGG
GIMAP6 $_{1-272}$ (r)	CGGCTCGAGGGACAGTCCTTCCAGCCA
GIMAP6 $_{1-282}$ (r)	CGGCTCGAGGTGGGCTTCCTCAGATTC
GIMAP6 $_{1-287}$ (r)	GCGCTCGAGTCACAGCAGGCATCTGTGGGC
GIMAP6 $_{L292A}$ (r)	GCGCTCGAGTCAAGCGTCAGCCTTCCCCAG
GIMAP6 $_{K289A}$ (r)	GCGGATGCTCGAGAAGGTCAGCCGCCCCCAGCA
GIMAP6 $_{HR283284AA}$ (r)	GCGCTCGAGAAGGTCAGCCTTCCCCAGCAGGCATGCGGCGGCTTC
GIMAP6 $_{R284A}$ (r)	GCGCTCGAGAAGGTCAGCCTTCCCCAGCAGGCATGCGTGGGCTT
GIMAP6 $_{H283A}$ (r)	CGCCTCGAGAAGGTCAGCCTTCCCCAGCAGGCATCTGGCGGCTTCCTC
GABARAPL2 (f)	GCTGGAATTCTGATGAAGTGGATG
GABARAPL2 (r)	GCGCTCGAGTCAGAAGCCAAAAGTGTT
GABARAPL2 $_{Y49A}$ (f)	GACAAACGGAAGGCCTTGGTTCCATCTGAT
GABARAPL2 $_{Y49A}$ (r)	ATCAGATGGAACCAAGGCCTTCCGTTTGTC
GABARAPL2 $_{L50A}$ (f)	GACAAACGGAAGTACGCGGTTCCATCTGAT
GABARAPL2 $_{L50A}$ (r)	ATCAGATGGAACCGCGTACTTCCGTTTGTC
GABARAPL2 $_{G116A}$ (r)	GCGCTCGAGTCAGAAGGCAAAAGTGTTCTC
GABARAPL2 $_{11-117}$ (f)	GCGGAATTCTGCTGGAACACAGATGCG
GABARAPL2 $_{25-117}$ (f)	GCGGAATTCTGTATCCCGACAGGGTTCCGG
Chimeric GABARAPL2-MAP1LC3B (f)	GCTGGAATTCTGATGAAGTGGATGTTCAAGG-AGGACCACTCGCGCACCTTCGAACAAAGAG
Chimeric GABARAPL2-MAP1LC3B (r)	GCGCTCGAGTTACACTGACAATTTCA

2.1.2 Agarose gel electrophoresis

DNA samples generated from PCR or digestion by restriction endonucleases were mixed with 1x DNA loading buffer for application onto a 1% (w/v) agarose gel containing 10 μg ethidium bromide in 0.5x TBE buffer. To separate the DNA fragments, gels were subjected to a constant voltage of 90 V for 45 min using a Bio-Rad POWERPAC 300 (Bio-Rad Laboratories Inc, USA) or until the dye front had migrated two-thirds of the way down the gel. DNA bands were then viewed using UV transillumination and photographed using a Gel Doc Imager.

2.1.3 Purification of PCR product

DNA amplified by PCR was purified using the QIAquick® PCR purification Kit (Qiagen, Germany) according to the manufacturer's instructions. This kit can purify up to 10 μg DNA of 100bp-10kb.

2.1.4 Measurement of nucleic acid concentration

DNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) using the 'Nucleic Acid' application module and DNA- 50 (double-stranded DNA) settings. Using a sample volume of 2 μL, the NanoDrop allows quantitative analysis of samples between 2-3700 ng/μL for double-stranded DNA.

2.1.5 Ligation

Ligations were performed using a 1:1/1:3/1:5 molar ratio of vector to insert, with 100 ng total DNA, 1 x T4 DNA ligase buffer and 1 unit of T4 DNA ligase (Invitrogen) in a total volume of 20 μL at 16 ºC overnight.

2.1.6 Preparation of LB (Luria-Bertani) agar plates

LB agar, prepared in-house, was melted in a microwave oven, then cooled to around 50 ºC before addition of antibiotics, typically ampicillin (0.1 mg/mL) or chloramphenicol (34 µg/mL). The LB agar was then poured into sterile plates aseptically and allowed to cool to room temperature. The plates were left to set and then were stored at 4 ºC until use.

2.1.7 Transformations

2.1.7.1 Using chemically competent bacteria

Heat shock transformation was performed. Briefly, the ligation mixture (20 µL) was added to 100 µL of *E.coli* DH5 alpha cells and chilled on ice for 30 min and mixed gently by tapping. The cells were then heat-shocked for 45 sec at 42 ºC before chilling on ice immediately. SOC medium (1ml, Invitrogen) was added to the cells which were then incubated with shaking (200 rpm) for 45 min. The transformed cells were then plated on pre-warmed LB agar plates containing appropriate antibiotic. The plates were incubated overnight at 37 ºC and then stored at 4 ºC.

2.1.7.2 Using electro-competent bacteria

For electroporation, PCR products or ligated DNA was added to electrocompetent bacteria, which was added to an electroporation cuvette with a 0.1 cm gap, on ice. The bacteria/DNA mix was electroporated at 1.80 kV and then placed directly into 500 µL SOC medium at 37 ºC for 1 h. An aliquot of bacterial suspension was spread onto an LB-agar plate containing appropriate antibiotics.

2.1.8 Isolation of plasmid DNA

For extraction of DNA, colonies were lifted off the agar plates using a sterile pipette tip and were grown up in 5ml LB containing the appropriate antibiotic, with shaking (200 rpm, 37 ºC, o/n). Plasmid DNA was isolated using the QIAprep® Spin Miniprep and Maxiprep Kits according to the manufacturer's instructions. DNA was eluted in 50 μL of ddH2O and DNA concentration measured. A diagnostic restriction digest with the appropriate restriction enzymes was carried out to screen for positive clones. An aliquot (between 100 and 500 ng) of the DNA was digested with 10 units of restriction enzyme in an appropriate buffer at 37 ºC for 1-4 h. The products were run on an agarose gel (subsection 2.1.2) and the DNA from the colonies giving correctly sized DNA fragments was sequenced (by Cogenics). Sequences were aligned with the BLAST2 sequence alignment tool, and bacterial clones containing the desired constructs were maintained as glycerol stocks at -80 ºC for long term storage.

2.2 Protein techniques

2.2.1 Preparation of cell lysates

Cells in suspension (Jurkat T cells) were collected by centrifugation at 400 x g, 4 ºC for 10 min while adherent cells (HEK293T cells) were harvested from plates with a sterile cell scraper. The cells were lysed by the addition of the appropriate lysis buffer (TX100 lysis buffer for pull-downs with streptavidin-agarose, SEC experiments and other routine experiments; GTP lysis buffer for pull-downs with GTP-agarose) containing a final concentration of 5 μL/mL of a protease inhibitor cocktail (Sigma P8340) and were incubated on ice for 20 min. The cell debris was removed by centrifugation at 20 000 x g, 4 ºC for 10 min. Supernatants were transferred to fresh microfuge tubes, and to an aliquot of the lysate, an equal volume of Laemmli sample buffer (Bio-Rad) containing 50 mM DTT was

added.

2.2.2 *In vitro* interaction between GST-GIMAP6 and GABARAPL2

This was carried out according to the protocol in Pascall et al., 2013. Briefly, full-length GIMAP6 cDNA fragment was transferred to plasmid pGEX4T-1 and transformed into the *E. coli* Rosetta strain (Novagen). A parallel transformation of plasmid pGEX4T-1 was also performed. 20 mL overnight Rosetta cultures in LB medium containing either the GST or the GST-GIMAP6 expression construct were used to inoculate 1 L pre-warmed LB medium containing 200 μg/mL ampicillin and 50 μg/mL chloramphenicol. Cells were grown at 37 ºC for 2-3 h to an OD_{600} of approximately 0.6. The cultures were then cooled to 16 ºC and protein expression induced by the addition of 0.1 mM IPTG. Cultures were then grown for a further 16 h at 16 ºC. All subsequent steps were carried out at 4 ºC. Bacteria were collected by centrifugation at 5000 g for 5 min, pellets washed in 20 mL PBS pH 7.3 and centrifuged as described above. The pellets were then re-suspended in 20 mL lysis buffer {PBS containing 5 mM EGTA, 1% (v/v) Triton X-100, 5 mM $MgCl_2$, pH 7.4 with 1:100 Bacterial Protease Inhibitor Cocktail (Sigma)} per pellet and cells were lysed by sonication. The resulting lysate was centrifuged at 10 000 g for 15 min and the supernatant applied to a 125 μL packed volume glutathione Sepharose 4B column previously pre-equilibrated in binding buffer (140 mM NaCl, 2.7 mM KCl, 1 mM DTT, 10 mM Na_2HPO_4, 10 mM KH_2PO_4 pH 7.3). The flow-through was collected and rebound to the column twice. The column was washed with 3 x 5 column volumes of binding buffer. Purified GABARAPL2 (20 μg) in 500 μL of binding buffer was added to the column and incubated for 1 h at 4 ºC. The column was washed as described above, and the bound protein (GST and GST-GIMAP6) was eluted by the addition of 2 x 100 μL of elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0). Eluates were

analysed by SDS-PAGE, and were then either stained with Coomassie Brilliant Blue R to assess recovery of GST/GST-GIMAP6 or by Western blotting to assay for the presence of GABARAPL2.

2.2.3 GTPγS loading assay

This assay was carried out in collaboration with Dr Heidi Welch (Babraham Institute). Glutathione beads with immobilised GST-tagged protein (GST, GIMAP6, or Rac) were washed in the assay buffer (20 mM Hepes, pH 7.5 at 4 ºC, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mg/mL fatty-acid free BSA, 1 mM DTT). To the washed beads assay buffer containing 100 µM GTPγS (tetralithium salt, from ICN, kept at -20 ºC) and 20 µCi [^{35}S]GTPγS (1250 Ci/mmol, from NEN) was added. The low Mg^{2+} concentration in the assay buffer helps to GTP-load the protein, as the GTPase nucleotide binding pocket requires Mg^{2+}. Aliquots of 50 µL containing EDTA at a final concentration of 2 mM or not and 2µCi [^{35}S]GTPγS were incubated for 30 min at 30 ºC. The reaction was stopped by adding ice-cold wash buffer (1xPBS, 1 mM EGTA, 10 mM $MgCl_2$, 1% Triton X-100, 0.1 mM GTP). The high Mg^{2+} concentration in the wash buffer inhibits further nucleotide exchange. The beads were washed with 8 x 400 µL ice-cold wash buffer and the beads recovered each time by centrifugation at 10 000 g for 15 s. After the final wash, the wash buffer was thoroughly aspirated and 400 µL Ultima Gold scintillation fluid (Packard) was added and vortexed well. The tubes with the beads were transferred into scintillation vials (Packard) and 5ml scintillation fluid was added. This mixture was vortexed well and [^{35}S]GTPγS loading was measured by β-scintillation counting.

2.2.4 Size exclusion chromatography

Lysates were harvested from HEK293T cells and Jurkat T cells as described in subsection 2.2.1. The lysate with a volume of 0.2 mL was applied on a Superdex 200 10/30 SEC column at

a flow rate of 1ml/min. The column was equilibrated with 2 column volumes (CV) of SEC buffer (20 mM Tris pH 7.5, 150 mM NaCl, 2 mM DTT). These experiments were carried out in collaboration with Michael Wilson (Babraham Institute). Fractions of interest were collected and analysed via western blotting.

2.2.5 SDS-PAGE

Cell lysates were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) following the protocol described by Kolbe et al. (1984). 10-12% (w/v) gels (30% acrylamide solution, 37.5:1 acrylamide:bis acrylamide) were prepared using the Mini-PROTEAN® 3 system (Bio-Rad Laboratories Inc, USA) and the resolving gels were overlaid with 200 μL of isopropanol to generate a flat gel interface. The isopropanol was washed off prior to the application of the 10% (w/v) polyacrylamide stacking gel. All samples added to the gels had been mixed 1:1 with 2x Laemmli sample buffer (Biorad; 35 mM DTT final) and denatured by heating at 95 ºC for 5 min. Precision Plus Protein Markers (BioRad) were run alongside the lysate samples to mark the molecular weights. The gels were subjected to a constant voltage of 90 V for 1.5 h. On completion of electrophoresis the proteins separated on the gel were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P) for analysis by immunoblots. Alternatively, proteins on the gel were detected by silver staining (Sigma) or Coomassie Blue staining (Gel Code Blue reagent, Pierce). For both silver and Coomassie Brilliant Blue staining, manufacturer's instructions were followed. For western blotting, the transfer was carried out using a semi-dry apparatus. The gels were subjected to a constant current of 4 mA for 80 min.

2.2.6 Immunoblotting

Following transfer, PVDF membranes were blocked (either o/n at 4 ºC, or 45 min at RT) in 5% milk powder in PBS-0.1% Tween-20 (hereafter referred to as blocking buffer). The

membrane was then probed with primary antibody in blocking buffer at RT for between 1 and 3h or overnight at 4 ºC. The membrane was then washed repeatedly in PBS-0.1% Tween-20. Following this, the membrane was probed with a secondary antibody conjugated to horseradish peroxidase for 1 h at RT. The membrane was again repeatedly washed in PBS-0.1% Tween-20 before being incubated with Pierce Super Signal or Millipore Immobilon enhanced chemiluminescence reagents for 5 min. The membrane was blotted dry again, was wrapped in Saran Wrap and was exposed to X-ray film (Amersham) which was developed in an Xograph Developer.

Table 2.2.1: Antibodies used for immunoblotting.

Antibodies are monoclonal unless stated otherwise.

Antibody	Clone	From	Working dilution	Source
Rat anti-hGIMAP1	MAC450	Tissue culture supernatant	1:10	Prepared in-house
Rabbit anti-hGIMAP2	-	Polyclonal antiserum	1:500	Kind gift from O. Daumke
Rat anti-hGIMAP4	MAC426	Tissue culture supernatant	1:10	Prepared in-house
Rat anti-hGIMAP5	MAC429	Tissue culture supernatant	1:10	Prepared in-house
Rat anti-hGIMAP6	MAC445	Tissue culture supernatant	1:10	Prepared in-house
Rat anti-hGIMAP7	MAC447	Tissue culture supernatant	1:10	Prepared in-house
Rat anti-mGIMAP6	MAC431	Tissue culture supernatant	1:10	Prepared in-house
Rat anti-hGABARAPL2	MAC446	Tissue culture supernatant	1:10	Prepared in-house
Rabbit anti-MAP1LC3B	-	Polyclonal antiserum	1:10	Sigma (L7543)
Mouse anti-β-actin	-	Ascitic fluid	1:10 000	Sigma (AC-15)
Mouse anti-myc (9E10)	-	Tissue culture supernatant	1:10	Prepared in-house
Goat anti-rat IgG HRP	-	-	1: 10 000	Jackson ImmunoResearch
Sheep anti-mouse IgG HRP	-	-	1:10 000	Amersham Bioscience
Goat anti-rabbit IgG HRP	-	-	1: 2000	DAKO Cytomation
Streptavidin-HRP conjugate	-	-	1:10 000	Calbiochem

2.3 Cell based methods

2.3.1 Cell line establishment and maintenance

2.3.1.1 Biot-GIMAP4/7-His myc-BirA Jurkat cell line

Jurkat T cells stably expressing either GIMAP4 or GIMAP7 were established following the protocol described in Pascall et al., 2013. Briefly, the myc-BirA-Jurkat cell line (Pascall et al., 2013) was maintained in RPMI (Invitrogen) supplemented with 10% FCS, 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen) and containing 500 μg/mL G418. Approximately 10^7 cells were transfected by electroporation with 20 μg of either PvuI-linearised plasmid biot-GIMAP4/7-His-pCAG-iPuro or the corresponding vector. Cells were allowed to recover overnight in complete medium containing 500 μg/mL G418. The following day, the cells were spun down and resuspended in 20 mL of complete medium containing 500 μg/mL G418 and plated at 100 μL/well into 96-well plates. The following day an equal volume of complete medium containing 500 μg/mL G418 and 6 μg/mL puromycin was added to each well. Every 3-4 days thereafter, half of the medium was replaced with fresh complete medium containing 500 μg/mL G418 and 3 μg/mL puromycin. Cells carrying the parental pCAG-iPuro vector were isolated in parallel, as controls. Biot-GIMAP4-His and Biot-GIMAP7-His expressing clones were identified by western blotting of cell lysates with a horseradish peroxidase-conjugated streptavidin probe. A single vector clone and a single clone carrying Biot-GIMAP4/7-His (termed the Biot-GIMAP4/7-His myc-BirA-Jurkat cell line) were maintained for subsequent analysis. Cells were maintained by the removal of an aliquot of cells and transferring to a new flask containing fresh medium (*in vitro* passage).

2.3.1.2 HEK293 cell lines expressing myc-tagged wt GIMAP and myc-tagged GIMAP variants

Cell lines were established following the protocol described in Pascall et al., 2013. HEK293 cells were plated in a 6-well plate and 24 h later were transfected using lipofectamine (Invitrogen) with a myc-tagged human GIMAP6-expressing plasmid or a myc-tagged plasmid expressing either $GIMAP6_{1\text{-}282}$, $GIMAP6_{G50D}$ or $GIMAP6_{S54N}$. The cells were trypsinised 24 h later and re-plated in a 10cm tissue culture plate. 48 hours after transfection, stably transfected cells were selected by growth in DMEM/10% fetal calf serum/100 units/mL penicillin/100 µg/mL streptomycin/800 µg/mL G418. Single colonies were screened for GIMAP6 expression by western blotting and immunofluorescence.

The cells were passaged when confluent by aspirating the medium and washing with sterile PBS. The cells were then detached by the addition of trypsin-EDTA (Invitrogen). The cells were resuspended in culture medium and were either used or, for maintenance, 10% of the cells were placed into a fresh flask/10 cm dish with fresh culture medium. To freeze the cells down, they were removed from the flask/dish as detailed for passaging. The cells were centrifuged and were resuspended in FCS containing 10% DMSO (Sigma), transferred to cryovials and frozen at -80 ºC in a freezing container (5100 Cryo 1 ºC Freezing Container, "Mr. Frosty", Nalgene). After at least 24 h the vials were transferred to liquid nitrogen.

2.3.2 Transient transfection of HEK293T cells

Cells were maintained in DMEM/10% (v/v) fetal calf serum/penicillin/streptomycin. Transfections were performed using either polyethyleneimine (Boussif et al., 1995) or lipofectamine (Invitrogen) according to the manufacturer's instructions and cells were analysed 48h post transfection. HEK293T cells that were approximately 40% confluent were transfected with 20 µg of plasmid DNA. Cell medium was replaced 14-16 h post transfec-

tion. Transfections were performed with plasmids biotGIMAP6 and biotGABARAPL2 which had been derived previously by PCR-based transfer of human GIMAP6 and GABARAPL2 from full-length cDNA clones BC074744 and BM544477, respectively, into the *EcoRI-XhoI* site of pcDNA3Biot1His6iresBirA (Pascall et al., 2013). Plasmids encoding N-terminally myc-tagged GIMAP proteins were derived by cloning the corresponding cDNA sequences into the *EcoRI-XhoI* site of pCANmyc3 (as pCANmyc1 (Saunders et al., 2009, 2010; Rubinfeld et al., 1996) but with a different reading frame downstream of the myc tag). Site-directed mutagenesis via PCR was performed on the above mentioned constructs to generate the variant GIMAP6 and GABARAPL2 proteins.

2.3.3 Induction of autophagy

The protocol described in Pascall et al., 2013 was followed. Amino acid starvation was employed. Briefly, cells to be starved were washed twice with starvation medium (140 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose, 20 mM HEPES pH 7.4, 1% (w/v) BSA) and then maintained in the same medium for the duration of the starvation.

2.3.4 Immunoprecipitations

Approximately 3 x 10^6 actively growing HEK293T cells were transfected with plasmids as indicated using polyethyleneimine. Approximately 24 hours later, cells were transferred to fresh medium. The following day, dishes were washed in PBS and lysed into the appropriate lysis buffer supplemented with mammalian protease inhibitor cocktail (Sigma-Aldrich) at 4 ºC for 10 min (subsection 2.2.1). The supernatant was clarified by centrifugation at 20,000 g for 10 min at 4 ºC. An aliquot of the supernatant (50 µL) was removed to an equal volume of 2 x CSB and boiled to represent a lysate sample. To the remaining supernatant was added either a 100 µL packed volume of streptavidin-agarose (for transfections including pcDNA3Biot1His6iresBirA-based expression plasmids) or

100 µL GTP-agarose beads. The suspensions were rotated at 4 ºC for 2h (streptavidin-agarose; Sigma-Aldrich), or for 2h-overnight (GTP-agarose; Sigma-Aldrich) and then centrifuged at 20 000 g for 30 s. The pellets were washed with 8 x 1ml lysis buffer and the beads recovered each time by centrifugation at 20 000 g for 30 s. The final washed pellets were resuspended in 50 µL 2 x CSB (and heated to 95 ºC for 5 min to elute the bound proteins. SDS-PAGE gel electrophoresis and western blotting of the samples were then performed as described previously (section 2.2), using antibodies as indicated.

2.3.5 Immunocytochemistry

Immunocytochemistry studies were performed following the protocol described in Pascall et al., 2013. Cells grown on glass coverslips were washed with PBS and then fixed by one of two methods:-

1. Cells were fixed in 4% (w/v) paraformaldehyde for 20 min at room temperature. Subsequently, coverslips were rinsed 2-3 times with DMEM and left shaking gently for 15 min to remove all traces of paraformaldehyde before subsequent processing. 2. Cells were fixed in ice-cold methanol (for staining with rabbit anti-MAP1LC3B antibody) for 1-2 min and then in 1% (w/v) bovine serum albumin (BSA) in PBS (BSA block) with shaking at room temperature for at least 1 h.

After the fixation and washing steps, cells were permeabilised by incubation with 0.1% (v/v) Triton X-100 in BSA block for a further 15 min at room temperature. The permeabilisation step was omitted for methanol fixed cells. The cells were then rinsed with 3 × 2ml PBS (total wash time 20 min) before incubation for 1 h with BSA to block non-specific sites. The cells were then incubated with primary antibodies diluted in 100 µL of BSA block for 30 min in the dark at room temperature. Cells were then rinsed with 4 × 2ml PBS before a 30 min incubation with secondary antibodies which were diluted in 100 µL BSA block. The cells were again rinsed with 4 × 2ml PBS to remove any excess anti-

bodies. VectaShield containing DAPI (Vector Laboratories Inc.) was then mounted onto the cells and a coverslip was carefully placed onto the glass slide covering the cells. Confocal images were captured using an Olympus FV1000 imaging system. Co-localisation of proteins was determined using Imaris software.

3 Molecular requirements of GIMAP6 for the interaction with GABARAPL2

3.1 Context

3.1.1 Interaction between GIMAP6 and GABARAPL2

While it has been shown that the GIMAP GTPases play important roles in the development and survival of lymphocytes (Barnes et al., 2010; Ciucci and Bosselut, 2014; MacMurray et al., 2002; Saunders et al., 2010; Schnell et al., 2006; Schulteis et al., 2008; Nitta et al., 2006; Yano et al., 2014; Hernandez-Hoyos et al., 1999), little is known of the molecular mechanisms by which they carry out their function(s). To gain an insight into the molecular mechanisms underlying the function of this family of small GTPases, our laboratory undertook a biochemical approach to identify *in vivo* binding partners for the GIMAPs. Employing a biotin-tag affinity approach combined with formaldehyde cross-linking to stabilise possible molecular interactions, GABARAPL2, a mammalian homologue of the yeast autophagy protein 8 (Atg8), was identified as a major and highly specific interacting partner of human (h) GIMAP6 (Pascall et al., 2013). GABARAPL2 is a ubiquitin-like protein that has been reported to be required for the autophagic pro-

cess: specifically it is believed to play a role in the maturation of the autophagosome (Weidberg et al., 2010). Our group has also shown that the normally cytosolic GIMAP6 re-localises to autophagosomes on induction of starvation-induced autophagy (Pascall et al., 2013). This chapter will focus on the structural basis of the molecular interaction between GIMAP6 and GABARAPL2 and investigate whether this interaction is required for the relocalisation of GIMAP6 to autophagosomes.

3.1.2 The Atg8 interacting motif (AIM) and the GTPase/AIG1 domain within GIMAP6

Autophagy was originally regarded as a non-selective, bulk degradation process that was primarily required for balancing sources of energy at critical times in development and in response to nutrient stress. Over the past decade, however, evidence has accumulated suggesting that autophagy can be highly selective and it has been shown to be responsible for the selective degradation of specific proteins and protein aggregates, organelles, and micro-organisms, amongst other cellular constituents (Lamark et al., 2009; Kirkin et al., 2009b,a). Selective autophagy is facilitated by the presence of selective autophagy receptors including SQSTM1, NDP52, Nix, and Optineurin that can bind specific cargo and interact with a member/s of the Atg8 family. For example, SQSTM1, the first selective autophagy receptor to be identified (Bjorkoy et al., 2005), interacts with MAP1LC3B and is a cargo receptor for the autophagic degradation of ubiquitylated protein aggregates (Pankiv et al., 2007). Abrogating autophagy by deleting the *Atg7* gene in mice, a gene required for autophagosome biogenesis, resulted in the failure of degradation of SQSTM1 and led to the accumulation of protein aggregates (Komatsu et al., 2007). Detailed deletion mapping combined with point mutation analyses has led to the identification of Atg8 interaction motifs (AIM) within the selective autophagy receptors. The consensus sequence of an AIM consists of acidic residues (D/E) N-terminal to a core [W/F/Y]xx[L/I/V] sequence

(Ichimura et al., 2008; Noda et al., 2008). Interestingly, hGIMAP6, but not mouse or rat GIMAP6, has a sequence of amino acids spanning positions 3-9 of the protein that corresponds to the canonical AIM. The putative AIM within GIMAP6 is composed of the following sequence of residues - EEE**Y**EQ**I.** I carried out point mutation analyses to investigate whether the putative AIM motif within GIMAP6 plays a role in its interaction with GABARAPL2.

The GIMAP family of proteins contain the well-conserved GTP-binding AIG1 domain and hence are considered to be GTPases. So far, however, the actual GTP binding ability and enzymatic activity of only hGIMAP2, hGIMAP7 and hGIMAP4 have been characterised. Prior to commencing my study, the GTP binding ability of GIMAP6 was unknown. It had also not been determined whether this domain played a role in the interaction with GABARAPL2. Assays to determine the GTP binding ability of GIMAP6 and point mutation analyses to analyse the requirement of the GTPase domain within GIMAP6 for its interaction with GABARAPL2 have been carried out.

3.2 Chapter Aims

1. Carry out studies to assess the GTP binding ability of hGIMAP6
2. Identify domains within GIMAP6 that are required for the interaction with GABARAPL2
3. Investigate whether the interaction between GIMAP6 and GABARAPL2 is required for the relocation of GIMAP6 to autophagosomes

3.3 Results

Prior to commencing my studies on GIMAP6 and its interaction with GABARAPL2, I attempted to discover novel interacting partners of hGIMAP4 and hGIMAP7 employing tag-mediated pull-downs as well as cross-linking. This search for novel interacting partners of GIMAP4 and GIMAP7 was performed by employing streptavidin affinity purification from Jurkat T cells. The Jurkat T cell line is derived from the peripheral blood of a patient with T-cell acute lymphoblastic leukaemia (T-ALL; Schneider et al., 1977) and was chosen as it is widely used to study T-cell signalling and, importantly, exhibits endogenous expression of hGIMAPs. Labelling proteins with biotin is widely used in order to take advantage of the strong and highly specific nature of the biotin-streptavidin interaction. The biotinylation reaction can be carried out by the BirA enzyme, a 35 kDa DNA-binding biotin protein ligase found in *E.coli* that catalyses the covalent addition of biotin to the lysine side-chain present within the 15 amino acid biotinylation tag that is fused to the target protein (Schatz, 1993; Drakas et al., 2005). A Jurkat T cell line stably transfected with the pmycBirA-ires-neo plasmid (referred to as the myc-BirA-Jurkat cell line henceforth) had been previously engineered in the laboratory. The myc-BirA-Jurkat cell line was engineered to stably over-express either GIMAP4 or GIMAP7 carrying a biotinylation target sequence. Cross-linking was performed using formaldehyde, disuccinimidyl suberate (DSS) (a homobifunctional lysine specific cross-linking reagent with a longer spacer arm than formaldehyde) and 1,6-bis-maleimidohexane (BMH) (a homobifunctional cross-linker that irreversibly conjugates sulfhydryl groups). Formaldehyde has a number of advantages in its use as a cross-linker: it is highly permeative, enabling cross-linking within the intact cell; its small size ensures that only closely associated proteins are cross-linked, and it is fast-acting, enabling rapid stabilisation of transient interactions. Unfortunately, for both GIMAP4 and GIMAP7 this strategy was unsuccessful due to the formation of high molecular weight protein aggregates, possibly due to excessive specific cross-linking. Additionally, no specific proteins appeared to co-purify with biotinylated

GIMAP4 or GIMAP7 after streptavidin affinity purification (results not shown).

3.3.1 Cross-linking using DSS confirms the presence of the GIMAP6-GABARAPL2 protein complex

GABARAPL2 was previously identified as an interacting partner of hGIMAP6 by using formaldehyde as a cross-linker to stabilise potential interactions, followed by mass spectrometry (Pascall et al., 2013). Further confirmation of the existence of this complex was performed by using DSS as a cross-linker. DSS is a water-insoluble, non-cleavable and membrane permeable cross-linker with an amine-reactive N-hydroxysuccinimide (NHS) ester at each end of an 8-carbon spacer arm. Jurkat T cell lines stably over-expressing a plasmid containing myc-tagged BirA and a plasmid carrying hGIMAP6 with an N-terminal biotinylation target sequence (Biot-GIMAP6-His myc-BirA), or the corresponding vector cell line (Biot-vector-His myc-BirA), that had been previously established in the laboratory were used and streptavidin affinity purification of a cross-linked biotinylated GIMAP6 protein complex was carried out. The cross-linking reaction was performed in cell lysates that were treated with a final concentration of 2 mM DSS for a duration of 1 h at RT. This was followed by streptavidin-affinity purification which was carried out by incubating lysates with the streptavidin-agarose beads for 2h at 4 ºC. Lysates were separated via SDS-PAGE and transferred to a PVDF membrane, following which immunoblots were probed with either a streptavidin-HRP conjugate or a rat monoclonal antibody (mAb) to GABARAPL2, MAC446, followed by an HRP-conjugated goat F(ab')$_2$ fragment anti-rat IgG to detect GABARAPL2. A discrete DSS cross-linked species corresponding to a relative mass (M_r) of ca. 55 kDa was detected when the membrane was probed with either the streptavidin-HRP conjugate or MAC446 (Figure 3.3.1). Biotinylated GIMAP6 exhibits a mobility on SDS-PAGE corresponding to an M_r of ca. 40 kDa while GABARAPL2 displays a mobility corresponding to M_r ca. 15 kDa. In the

cross-linked sample, however, both the streptavidin-HRP conjugate as well as MAC446 detected the presence of a cross-linked species corresponding to an M_r of ca. 55 kDa. As MAC446 was also able to detect a species of the same M_r , the presence of a complex containing GIMAP6 and GABARAPL2 was confirmed. Furthermore, GABARAPL2 was detected at a much lower intensity by MAC446 at its expected molecular mass in the cross-linked sample consistent with its presence in the ca. 55 kDa complex.

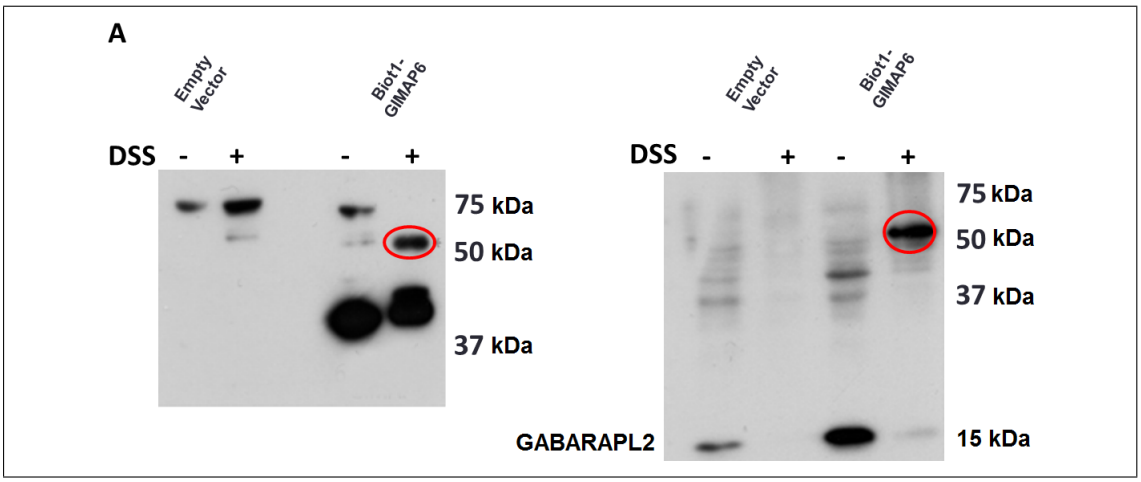

Figure 3.3.1: GIMAP6 is cross-linked to GABARAPL2 when DSS is used as a cross-linker.
Jurkat T cells engineered to over-express myc-tagged BirA and GIMAP6 carrying a biotinylation target sequence were incubated with or without 2 mM DSS for 1 h at RT. Cell lysates were analysed by immunoblotting with either (A) the streptavidin-HRP conjugate to detect biotinylated proteins or (B) the rat monoclonal antibody to GABARAPL2, MAC446 followed by an HRP-conjugated goat F(ab')$_2$ fragment anti-rat IgG to detect GABARAPL2. The mobilities of the biotinylated GIMAP6 and the cross-linked species are shown. The cross-linked species is within the red circle in both A and B. In figure A, proteins with an electrophoretic mobility of approx. 75 kDa are believed to endogenous proteins biotinylated proteins by BirA. In Figure B, the reason behind the presence of the proteins in lanes 1 and 3 displaying mobilities between 37 kDa and 75 kDa is not understood. These may be non-specific in nature.

The streptavidin-HRP conjugate was able to detect biotinylated GIMAP6 at its expected molecular mass, albeit at a lower relative intensity, in the cross-linked sample. The size of the individual components of the GIMAP6-GABARAPL2 complex (ca. 40 kDa and ca. 15 kDa, respectively), and the size of the cross-linked complex (ca. 55 kDa) strongly

suggested that the interaction between GIMAP6 and GABARAPL2 was direct. This hypothesis was tested and the findings are shown later on in the thesis. The ca. 55 kDa complex was absent in samples that were not cross-linked and was also absent in lysate samples prepared from the Biot-vector-His myc-BirA Jurkat T cell line.

3.3.2 The GIMAP6-GABARAPL2 interaction can be detected without cross-linking

To investigate whether the GIMAP6-GABARAPL2 interaction could be detected without cross-linking, I assessed the ability of hGIMAP6, when over-expressed either in transiently transfected HEK293T cells or in the Biot-GIMAP6-His myc-BirA Jurkat T cell line, to interact with endogenous GABARAPL2. HEK293T cells were transfected with a plasmid in which wild-type (wt) *GIMAP6* was cloned into the pcDNA3 vector carrying a CMV promoter, an N-terminal biotinylation tag and the internal ribosome entry site (IRES) sequence followed by *BirA* (Biot1-GIMAP6-His6-IRES-BirA). The IRES enables the bicistronic expression of wt GIMAP6 and BirA. This plasmid (20 μg) was transfected in HEK293T cells and lysates were prepared 48h post transfection. Lysates were also prepared from the Biot-GIMAP6-His myc-BirA Jurkat T cell line, or the corresponding vector-only cell line and biotinylated and associated proteins were recovered by streptavidin-agarose affinity purification. Lysates were separated via SDS-PAGE and transferred to a PVDF membrane and immunoblots were probed with either the streptavidin-HRP conjugate or MAC446. Immunoblots showed the recovery of biotinylated GIMAP6 and the co-precipitation of endogenous GABARAPL2. Co-precipitation of GABARAPL2 is not seen in cells (both HEK293T and Jurkat T cells) carrying the vector without GIMAP6 (Figure 3.3.2). Unfortunately this interaction could not be detected between endogenous GIMAP6 and endogenous GABARAPL2 (Pascall et al., 2013), possibly due to either the transient nature of the interaction or the inability of the available

antibodies to immunoprecipitate or to recognise the complex.

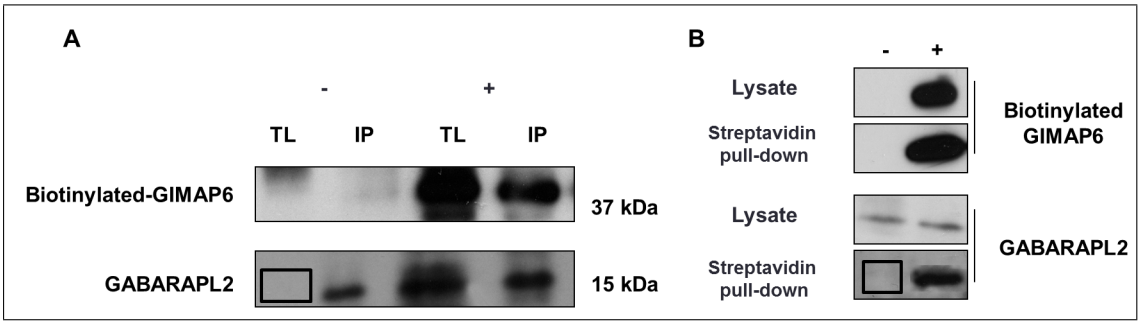

Figure 3.3.2: The GIMAP6-GABARAPL2 interaction can be detected without cross-linking.
A: Immunoblots carried out on lysates obtained from HEK293T cells that were transiently transfected with wt GIMAP6 in plasmid pcDNA3Biot1His6iresBirA (+), or the corresponding vector (-). SP: streptavidin pull-down. B: Immunoblots carried out on lysates obtained from Jurkat T cells that were either stably transfected to over-express myc-BirA and GIMAP6 carrying a biotinylation target sequence (+) or engineered to stably express myc-BirA and the corresponding vector (-). Biotinylated and associated proteins were recovered by streptavidin-agarose affinity purification (48h post transfection in the case of HEK293T cells). Immunoblots of the recovered proteins were probed with the streptavidin-HRP conjugate to detect GIMAP6 proteins or the rat monoclonal antibody MAC446 to GABARAPL2 followed by an HRP-conjugated goat $F(ab')_2$ fragment anti-rat IgG.

3.3.3 The role of the AIM in the interaction of GIMAP6 with GABARAPL2

As introduced in subsection 3.1.2, the N-terminus of hGIMAP6 contains within it a canonical putative AIM sequence spanning amino acids 3-9 (EEEYEQI). Constructs were designed to: (i) substitute Y6 and, separately, I9, to A. The Y and I residues of the AIM are believed to bind the hydrophobic pockets 1 and 2, respectively, within the core ubiquitin-like domain of GABARAPL2 (Noda et al., 2010, 2008; Ichimura et al., 2000). These mutant GIMAP6 sequences were generated via PCR mutagenesis and were cloned into the Biot1-His6-IRES-BirA plasmid. HEK293T cells were transfected with these constructs and lysates were prepared 48h post transfection. Streptavidin affinity purification

was performed and the recovered biotinylated and associated proteins were detected as described in the legend to Figure 3.3.3. As shown in the figure, the putative AIM is not required for the interaction of GIMAP6 with GABARAPL2 as the latter is seen to co-precipitate with the variant GIMAP proteins. The GIMAP6 amino acid sequence was further inspected to attempt to identify additional regions within the protein that may correspond to an AIM. No such sequence could be identified.

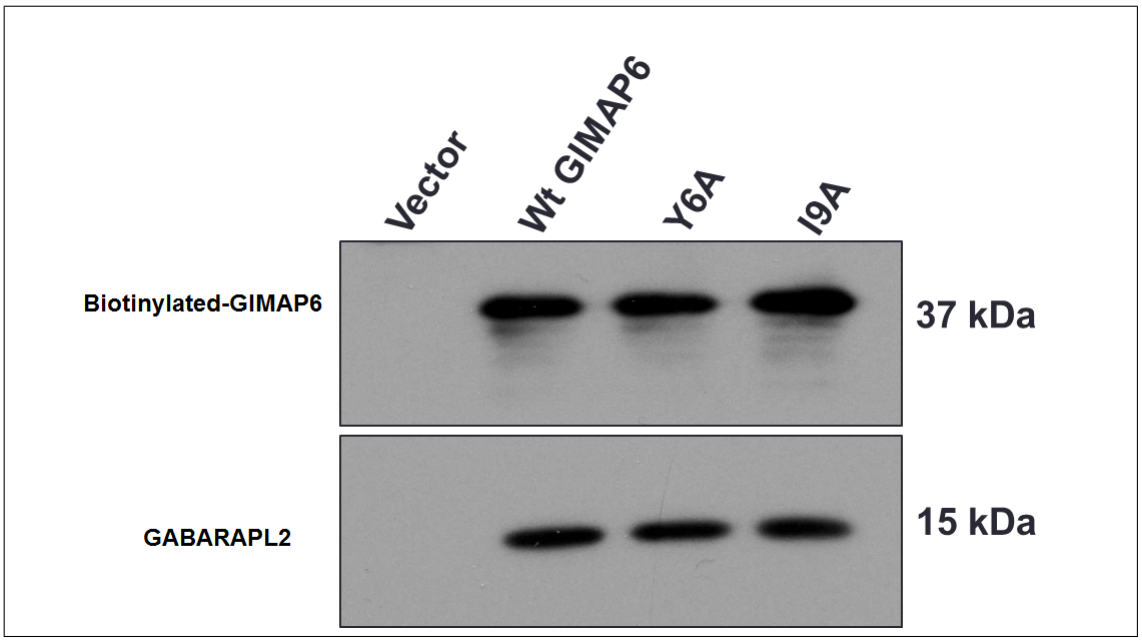

Figure 3.3.3: The putative AIM within the N-terminal region of GIMAP6 is not required for the GIMAP6-GABARAPL2 interaction.
HEK293T cells were transfected with wt GIMAP6 or the indicated mutated derivatives in plasmid pcDNA3Biot1His6iresBirA. Biotinylated and associated proteins were recovered by streptavidin-agarose affinity purification 48h post transfection. Immunoblots of the recovered proteins were probed with the streptavidin-HRP conjugate to detect the GIMAP6 proteins (upper panel) or the rat monoclonal antibody MAC446 followed by an HRP-conjugated goat F(ab')$_2$ fragment anti-rat IgG to detect GABARAPL2 (lower panel).

3.3.4 Role of the GTPase AIG1 domain within GIMAP6 in its interaction with GABARAPL2

As mentioned in subsection 3.1.2, the GIMAP family contains the highly conserved GTP binding AIG1 domain. Prior to investigating whether this region played a role in interacting with GABARAPL2, it was pertinent to ask whether GIMAP6 is indeed able to bind GTP. The nucleotide binding and hydrolytic activity of GTPases is commonly characterised by generating purified protein and employing radioactive [^{35}S]GTPγS and [α^{32}P]GTP or [γ^{32}P]GTP. Measuring the amount of GTP bound to the protein is possible when the assay is carried out with GTPγS, the slowly hydrolysable analogue of GTP. The catalytic activity can be analysed by using the isotopically-labelled native GTP and measuring the amount of either GDP or radioactive phosphate released. The *GIMAP6* gene was inserted into the bacterial expression vector pGEX-4T-1 and *Escherichia coli* (*E.coli*) Rosetta DE(3) bacteria (Novagen) were transformed with this construct, enabling IPTG-inducible expression of a fusion protein with the glutathione-S-transferase (GST) tag N-terminal to GIMAP6. Expression of GST-GIMAP6 was induced by the addition of 100 μM IPTG when cultures that had reached the exponential phase of growth had been cooled to 16 ºC. Subsequent to the addition of IPTG, an overnight incubation of the cultures was carried out at 16 ºC. A low temperature was chosen for induction of protein expression as this has been shown in other cases to enhance the yield of active soluble protein (Coligan et al., 2001). Attempts made to purify GST-GIMAP6 by solubilising the pellet in *E.coli* lysis buffer showed that the majority of the protein appeared to accumulate as insoluble aggregates (inclusion bodies) (Figure 3.3.4).

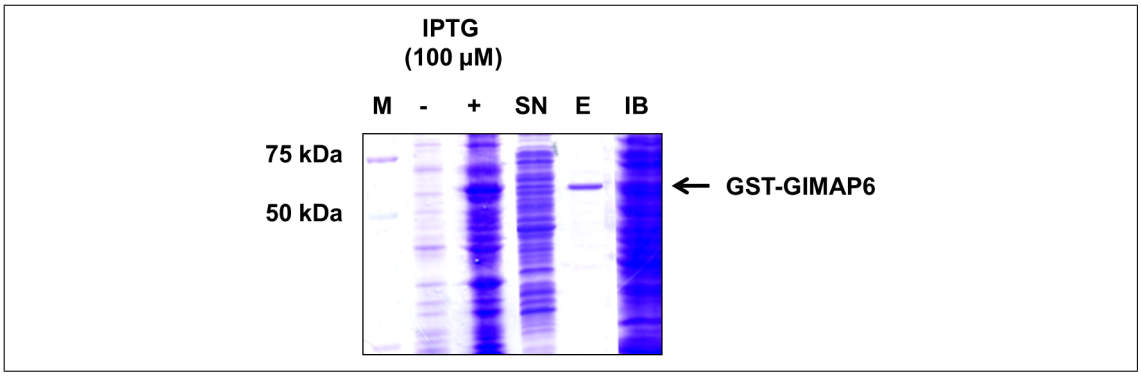

Figure 3.3.4: GST-GIMAP6: bacterial expression and purification.
Various samples taken during the purification were resolved by SDS-PAGE; proteins were subsequently visualised by Coomassie Blue staining. M: molecular weight marker; -/+ IPTG (final concentration 0.1 mM), whole-cell bacterial lysates before and after overnight induction at 16 ºC; SN: supernatant of bacterial lysates after centrifugation (20000 g, 10 min, 4 ºC); E: GST-GIMAP6 eluted with glutathione; IB: sample extracted from bacterial pellet suspended in 6M urea.

The small proportion of GST-GIMAP6 that appeared soluble was affinity purified using glutathione-Sepharose beads. Assays to characterise whether GIMAP6 could bind GTP were carried out by loading [^{35}S]GTPγS on to GST-GIMAP6 that was immobilised on the glutathione-Sepharose beads. Purified GST-Rac that had been immobilised on glutathione-Sepharose beads (a kind gift from Dr. Heidi Welch, Babraham Institute) was used as a positive control. The experiment was carried out in the presence or absence of EDTA. EDTA chelates the Mg^{2+} ions which would allow the [^{35}S]GTPγS to be loaded. [^{35}S]GTPγS-loading of GST-Rac, GST and GST-GIMAP6 was quantitated by β-scintillation counting. While GST-Rac was able to load [^{35}S]GTPγS, counts generated by GST-GIMAP6 were very similar to those generated by the no protein negative control (Figure 3.3.5). Inconclusive results were also obtained when GST-GIMAP6 was eluted off the beads, concentrated, and then loaded with [^{35}S]GTPγS (not shown). The technical hurdles in obtaining active soluble GIMAP6 have also been encountered by a research group in Germany working on the GIMAP GTPases (personal communication, O. Daumke). They have also observed that GIMAP6 has a strong propensity to aggregate, thereby posing a significant hurdle in the biochemical characterisation of purified

GIMAP6.

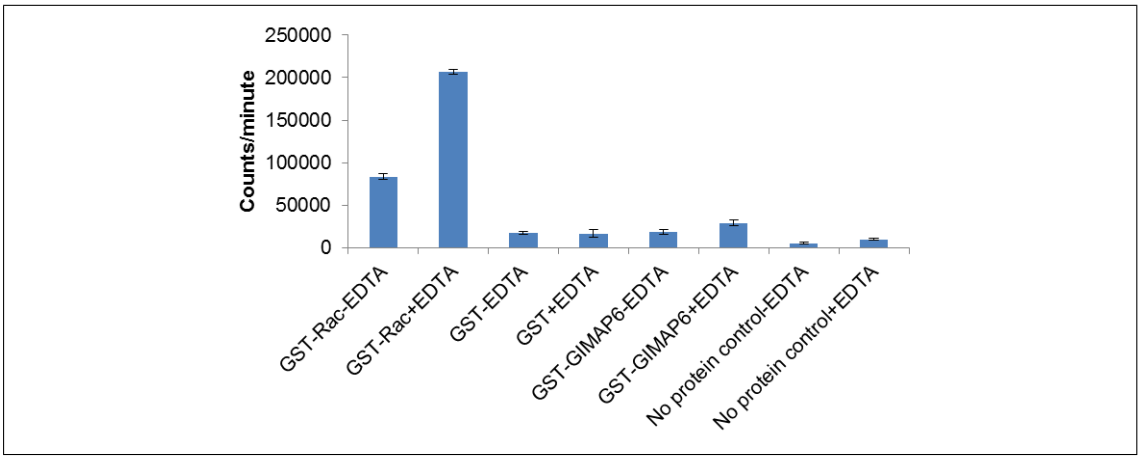

Figure 3.3.5: [^{35}S]GTPγS loading of GST-GIMAP6.
GST, GST-GIMAP6 or GST-Rac immobilised on glutathione beads were used for this assay. The beads were incubated with 10 µM GTPγS and and 2µCi [^{35}S]GTPγS for 30 min at 30 ºC in the presence (or not) of EDTA at a final concentration of 2 mM. The reaction was stopped by adding ice-cold wash buffer (subsection 2.2.3). To the beads 400 µL Ultima Gold scintillation fluid was added and vortexed. The tubes with the beads were transferred into scintillation vials and scintillation fluid was added. [^{35}S]GTPγS loading was measured by β-scintillation counting. Technical replicates done in duplicates. Error bars represent standard error of mean.

3.3.4.1 GTP-agarose beads

An alternate approach to answering the question of whether a putative GTPase can in fact bind GTP/GDP is the use of GTP-agarose beads (Carlessi et al., 2011; Jebelli et al., 2012). While characterising nucleotide binding ability using purified protein is the canonical approach, for proteins that are prone to aggregation, the use of GTP-agarose beads can provide a viable approach to determine, in general terms, their nucleotide binding abilities. It is advantageous in this respect that the beads can be used to interrogate crude cell lysates in which the target protein (identifiable by means of a specific antibody or generic epitope tag) may have a greater opportunity to retain its native, undenatured conformation. In this approach, the beads can be incubated with either lysates from cells that endogenously express the protein in question or lysates from cells that have been engin-

eered to over-express the protein or its variants. As Jurkat T cells express the GIMAP family of proteins endogenously, they were used for this assay. Aliquots of Jurkat T cell lysate were incubated overnight at 4 ºC with the GTP-agarose beads in the presence or absence of free GDP or GTP. Western blots of bound proteins probed with rat anti-human GIMAP6 monoclonal antibody MAC445 showed that GIMAP6 can bind the GTP-agarose beads and that this interaction could be efficiently abrogated by the addition of increasing concentrations of free GTP or GDP. GDP was less efficient than GTP at competing with the interaction between GIMAP6 and the GTP-agarose beads, indicating that GIMAP6 has a higher affinity for GTP than for GDP (Figure 3.3.6). To demonstrate specificity of the GIMAP6-GDP/GTP interaction, lysate from Jurkat T cells was incubated with the GTP-agarose beads in the presence of free ATP. The free ATP did not have any measurable effect on the interaction between GIMAP6 and the GTP-agarose beads.

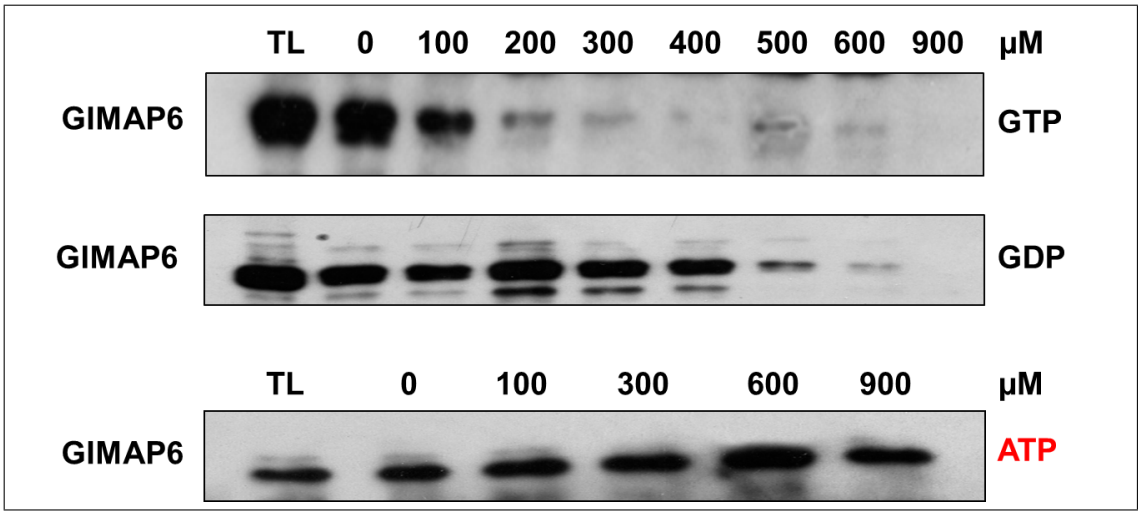

Figure 3.3.6: GIMAP6 binds GTP
Lysates from wt Jurkat T cells were incubated overnight with GTP-agarose beads at 4 ºC in the presence of increasing concentrations of GTP, GDP or ATP. After incubation beads were washed with GTP lysis buffer to wash away unbound proteins. Western blots were carried out on the extracts from 10^5cells in the Total Lysate (TL) lane, and on the extract from $6x10^5$ cells in the other lanes, indicating a recovery of approx. 15% of GIMAP6 following incubation with GTP-agarose beads when the samples are treated with vehicle (PBS; 0 µM lane). Immunoblots were probed with MAC445, followed by an HRP-conjugated goat F(ab')$_2$ fragment anti-rat IgG, to detect GIMAP6.

Next, to analyse the dynamics of GIMAP6 binding to the GTP-agarose beads, aliquots of the lysate from Jurkat T cells were incubated with the GTP-agarose beads for a duration ranging from 2h to an overnight incubation at 4 ºC, with or without the inclusion of 1 mM free GTP. Immunoblots indicated that a 2h incubation was sufficient for GIMAP6 to bind the GTP-agarose beads, and that this interaction reached its maximum level after about 8h incubation at 4 ºC, after which time-point no significant increase in binding efficiency was seen (Figure 3.3.7). The length of the incubation required may suggest a slow on-rate. The results in this section support the view that GIMAP6 is a bona fide GTP binding protein and that therefore it is valid to consider its functional activity in this context.

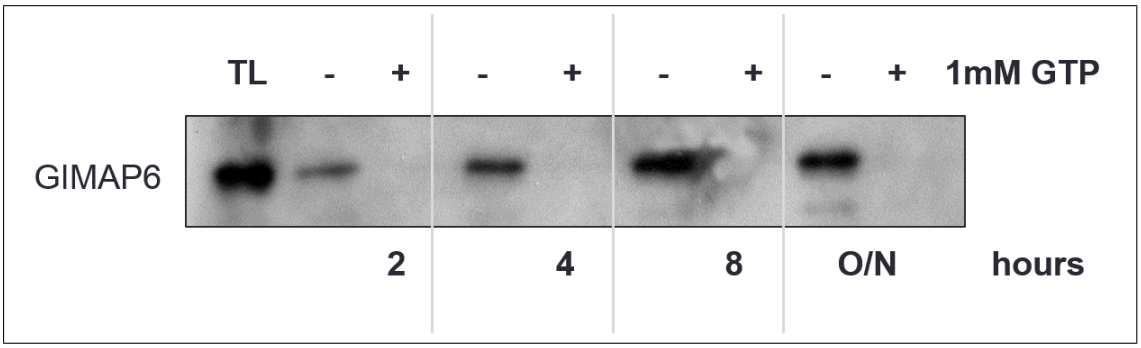

Figure 3.3.7: Dynamics of GIMAP6 binding to GTP.
Lysates from wt Jurkat T cells were incubated with GTP-agarose beads at 4 ºC for durations ranging from 2h to overnight in the presence or absence of 1 mM free GTP. After incubation, beads were washed with GTP lysis buffer to wash away unbound proteins. Western blots were carried out on lysates from 10^5cells in the Total Lysate (TL) lane, and on the extract from $6x10^5$ cells in the other lanes. Immunoblots were probed with MAC445, followed by an HRP-conjugated goat $F(ab')_2$ fragment anti-rat IgG to detect GIMAP6. The Jurkat T cell lysate needs to be incubated with the GTP-agarose beads for at least 8 h to obtain an efficient recovery of GIMAP6.

3.3.4.2 The effect of mutations within the AIG1/GTP binding domain of GIMAP6 on its interaction with GABARAPL2

Data from the above experiments suggest that GIMAP6 is a bona fide guanine nucleotide binding protein, if not an active catalytic GTPase. This raised the question of whether the AIG1 domain of GIMAP6 is functionally required for its interaction with GABARAPL2.

Magnesium ions (Mg^{2+}) play an important role in the nucleotide binding and catalytic activity of GTPases (Bourne et al., 1991); they force the triphosphate into a stretched conformation in which the β- and γ-phosphates are coordinated in a manner that allows for hydrolysis (Rudack et al., 2012). A preliminary experiment using HEK293T cell lysates was therefore carried out in which Mg^{2+} ions were chelated. HEK293T cells were transiently transfected with biotin-tagged GIMAP6 and the lysates (prepared as described in subsection 3.3.2) were treated with 15 mM EDTA or EGTA and streptavidin-agarose affinity purification was carried out as described in subsection 3.3.2. EDTA chelates divalent cations and has a high affinity for Mg^{2+} while EGTA has a lower affinity for Mg^{2+} but a high affinity for, e.g., Ca^{2+} ions, thus serving as a control in these experiments. Chelation of Mg^{2+} would be expected to disrupt the GTP binding by GIMAP6. Immunoblots show the recovery of biotinylated GIMAP6, and show that the co-purification of endogenous GABARAPL2 is significantly affected by EDTA treatment, but not upon EGTA treatment. This observation is consistent with the idea that the GTPase domain within GIMAP6 has a role in the interaction with GABARAPL2 (Figure 3.3.8).

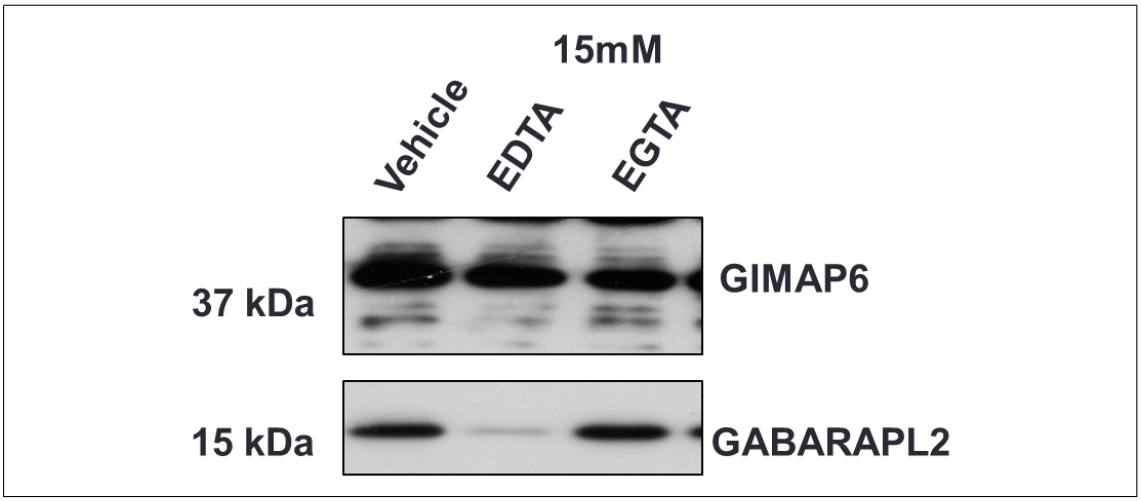

Figure 3.3.8: Chelation of Mg^{2+}adversely affects the GIMAP6-GABARAPL2 interaction.
Cell lysates were prepared from HEK293T cells that had been transiently transfected with wt GIMAP6 in the pcDNA3 Biot1-His6-IRES-BirA plasmid for 48h. Lysates were treated with vehicle (PBS), 15 mM EDTA, or 15 mM EGTA and biotinylated proteins along with associated proteins were recovered by streptavidin-affinity chromatography. Immunoblots of the recovered proteins were probed with the streptavidin-HRP conjugate (to show the GIMAP6 proteins; upper panel) or rat monoclonal antibody MAC446 to GABARAPL2 followed by an HRP-conjugated goat $F(ab')_2$ fragment anti-rat IgG (lower panel) to demonstrate co-precipitation of GABARAPL2. Treating lysate with EDTA, but not EGTA, affected the GIMAP6-GABARAPL2 interaction, pointing to a role for the GTPase domain within GIMAP6 in the interaction.

Selected mutations were introduced by mutagenic PCR into the G motifs (G1-G5; refer introduction) of the GTPase/AIG1 domain within GIMAP6 (Figure 3.3.9). These mutant *GIMAP6* sequences were cloned into the Biot1-His6-IRES-BirA plasmid and then HEK293T cells were transfected as detailed above. Introduction of mutations within the predicted G1 (G50D and S54N), G2 (T76A), G3 (D95A and N98A) and G5 (N201A) motifs prevented the GIMAP6-GABARAPL2 interaction.

Figure 3.3.9: A representation of the motifs within the GTP binding domains of hGIMAPs.

Fully conserved amino acid residues are shown in red, and residues that are at least 60% conserved in green. The three consecutive G domains of GIMAP8 were aligned individually. β-strands are shown as green arrows, α-helices as red barrels and loops as black lines. The G motifs involved in nucleotide binding and the switch I and II regions are indicated. The C-terminal hydrophobic segments of GIMAP1, 2 and 5 are boxed. Black stars indicate residues that were substituted. Figure adapted from Schwefel et al., 2013.

Mutations were introduced at these particular sites based on the conserved nature of these residues within the Ras superfamily of GTPases (refer section 1.3). The G50D mutation within the G1 motif of GIMAP6 is postulated to result in a gain of function variant of GIMAP6 that is constitutively bound to GTP and can interact with downstream effectors. The S54N variant in G1 is hypothesised to result in a dominant-negative variant that is unable to bind GTP, but can bind GDP. The T76A mutation within the G2 motif is believed to be constitutively bound to GTP, but unable to signal, while mutations within the G3 and G5 motifs affect nucleotide binding (Feig, 1999; García-Mata et al., 2006; Cherfils and Chardin, 1999).

The G4 motif normally determines selectivity for guanine nucleotides over adenine nucleotides. Surprisingly, introducing a mutation within the putative G4 motif of GIMAP6 by mutating the conserved aspartic acid to alanine (D167A) did not affect the interaction between GIMAP6 and GABARAPL2 (Figure 3.3.10). Structural analyses have revealed that the GIMAP family displays some structural similarity to the dynamin family of proteins (Schwefel et al., 2010) and, interestingly, mutating the conserved aspartic acid in dynamin I (DymA) did not significantly affect nucleotide selectivity or nucleotide binding (McMahon, 2004). One possible reason for the finding seen with the $GIMAP6_{D167A}$ variant could be that the role played by the aspartic acid residue in the selective recognition of the guanine base in Ras may be taken on by a separate, as yet unidentified, residue.

The arginine at position 134 was also mutated to alanine, as recent work on the structure of GIMAP2 has shown that the corresponding arginine (117) plays a role in its homodimerisation and this associated with GTP binding (Schwefel et al., 2010). Mutating this residue within GIMAP6 did not appear to affect the GIMAP6-GABARAPL2 interaction (Figure 3.3.10).

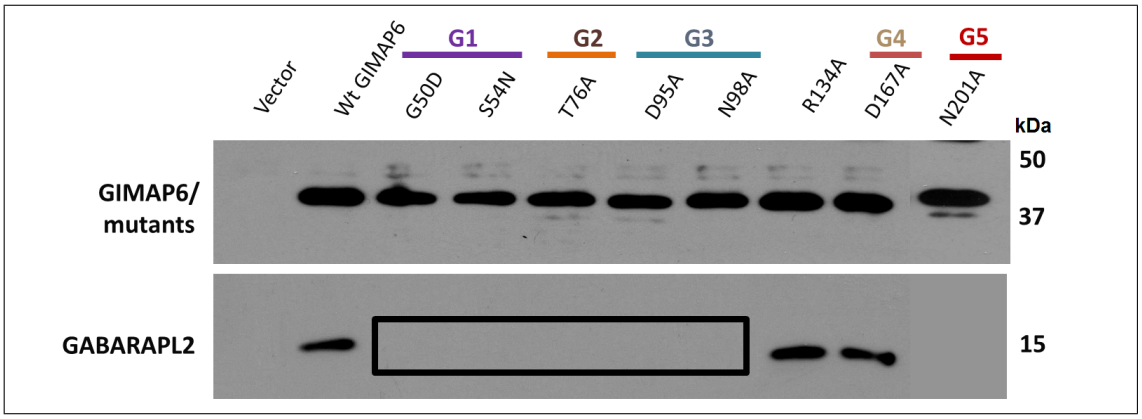

Figure 3.3.10: Mutations within the GTPase domain of GIMAP6 abrogate the interaction with GABARAPL2.
HEK293T cells were transfected with wt GIMAP6 or the indicated mutated derivatives in plasmid pcDNA3Biot1His6iresBirA. Biotinylated and associated proteins were recovered by streptavidin-agarose affinity purification 48h post transfection. Immunoblots of the recovered proteins were probed with the streptavidin-HRP conjugate to detect the GIMAP6 proteins (upper panel) or rat monoclonal antibody MAC446 to detect GABARAPL2 followed by an HRP-conjugated goat F(ab')$_2$ fragment anti-rat IgG (lower panel).

I then tested whether mutations within the GTPase domain of GIMAP6 affected the ability of the GIMAP6 variants to interact with the GTP-agarose beads and whether GIMAP6 variants that could interact with GABARAPL2 could still bind GTP-agarose. For this, either HEK293 cells were engineered to stably over-express myc-tagged GIMAP6$_{G50D}$ or GIMAP6$_{S54N}$, or HEK293T cells were transiently transfected with the Biot1-His6-IRES-BirA plasmid with either *GIMAP6*$_{S54N}$, *GIMAP6*$_{37\text{-}292}$ (subsection 3.3.5), or *GIMAP6*$_{R134A}$ inserted into the plasmid. Lysates were prepared from the stable HEK293 cell lines when the cells reached a confluency of roughly 80-85%, while lysates from the transiently transfected HEK293T cells were prepared 48h post transfection. The lysates were then incubated with the GTP-agarose beads overnight at 4 ºC as detailed above. Proteins were eluted from the beads and western blotting carried out with MAC445. Mutations within the G1 motif of the GTPase domain abrogated binding to the GTP-agarose beads, indicating that these variants had lost their ability to bind GTP (Figure 3.3.11; Left panel). Mutants that were known to be able to interact with GABARAPL2 (*GIMAP6*$_{37\text{-}292}$ (subsection 3.3.5)

and *GIMAP6*$_{R134A}$) are able also to bind GTP-agarose suggesting that an intact GTP binding ability may be essential for the GIMAP6-GABARAPL2 interaction (Figure 3.3.11; Right Panel).

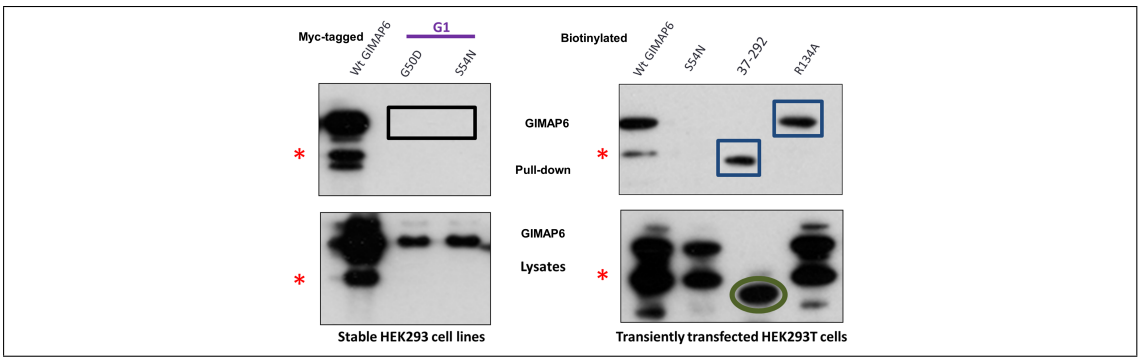

Figure 3.3.11: Analysis of some GIMAP6 mutants for binding to GTP-agarose. Left panel; Cell lysates were prepared from HEK293 cells engineered to stably express myc-tagged GIMAP6$_{G50D}$ or GIMAP6$_{S54N}$. Right panel; HEK293T cells were transfected with biotin-tagged GIMAP6 or the indicated mutated derivatives in plasmid pcDNA3Biot1His6iresBirA. GIMAP6 or the variants were recovered (or not) by GTP-agarose affinity chromatography 48h post transfection. Immunoblots of the recovered proteins were probed with MAC445 (anti-human GIMAP6) followed by an HRP-conjugated goat F(ab')$_2$ fragment anti-rat IgG (lower panel). Mutations within the G1 motif of the GTPase domain of GIMAP6 abrogate binding to GTP-agarose, while the two GIMAP6 variants that can interact with GABARAPL2 are able to bind GTP-agarose. Red asterisk denotes possible GIMAP6 breakdown product; green circle denotes GIMAP6$_{37\text{-}292}$variant.

Could the active GTPase cycle of GIMAP6 be dependent on its ability to interact with GABARAPL2? Jurkat T cells engineered to express the tetracycline repressor protein (T-REx^{TM} Jurkats) that were stably transfected with GABARAPL2 shRNA sequences were employed to address this question. Expression of the shRNAs is induced upon treatment with doxycycline. Cells were treated with either 1 μg/mL doxycycline for 4 days or maintained without doxycycline. Consistent with the data in (Pascall et al., 2013), a knockdown in the expression of GABARAPL2 is observed when the expression of the shRNA against GABARAPL2 is induced (Figure 3.3.12). Interestingly, a downregulation in the expression of GIMAP6 is also observed (Figure 5.2.2). Downregulation of GABARAPL2 appears to have some effect on the efficiency of the pull-down, however,

this could be due to the reduction in GIMAP6 levels that is observed when GABARAPL2 is knocked down. Data shown in this figure is inconclusive and not aided by the apparent mutual stabilisation of GIMAP6/GABARAPL2 levels.

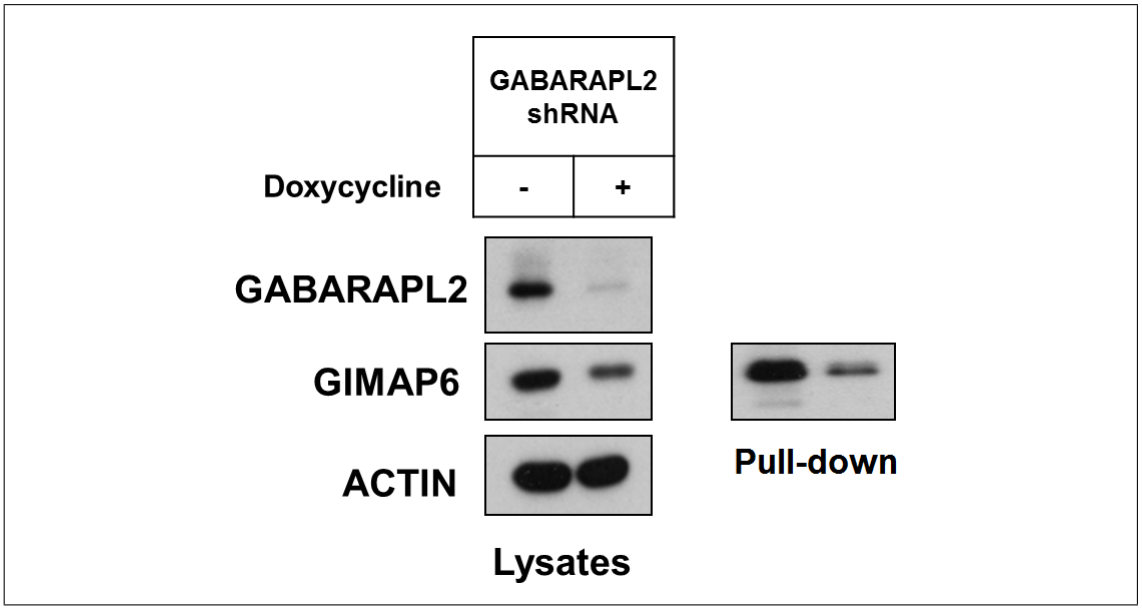

Figure 3.3.12: Effect of the knockdown of GABARAPL2 on the ability of GIMAP6 to bind to GTP-agarose.
Jurkat T-REx^{TM} cells carrying GABARAPL2 shRNA sequences were either treated for 4 days with 1µg/mL doxycycline or were similarly maintained in the absence of doxycycline. Cell lysates were prepared and aliquots were assayed by western blotting for GIMAP6 (using monoclonal antibody MAC445), GABARAPL2 (using monoclonal antibody MAC446), or β-ACTIN followed by the appropriate HRP-conjugated secondary antibodies. The remainder of the lysates were incubated overnight with GTP-agarose beads at 4 ºC. After incubation beads were washed with GTP lysis buffer to wash away unbound proteins. Immunoblots were probed with MAC445, followed by an HRP-conjugated goat F(ab')$_2$ fragment anti-rat IgG to detect GIMAP6.

3.3.5 The C-terminal domain of GIMAP6 is required for its interaction with GABARAPL2

While there is a high level of conservation within the GTPase/AIG1 domain amongst the mammalian GIMAP6 orthologues, the regions outside this domain show more divergence. An interesting observation is that three rodent species (rat, mouse, chinese hamster), but

not the mole rat, have divergent C-terminal domains (Figure 3.3.13).

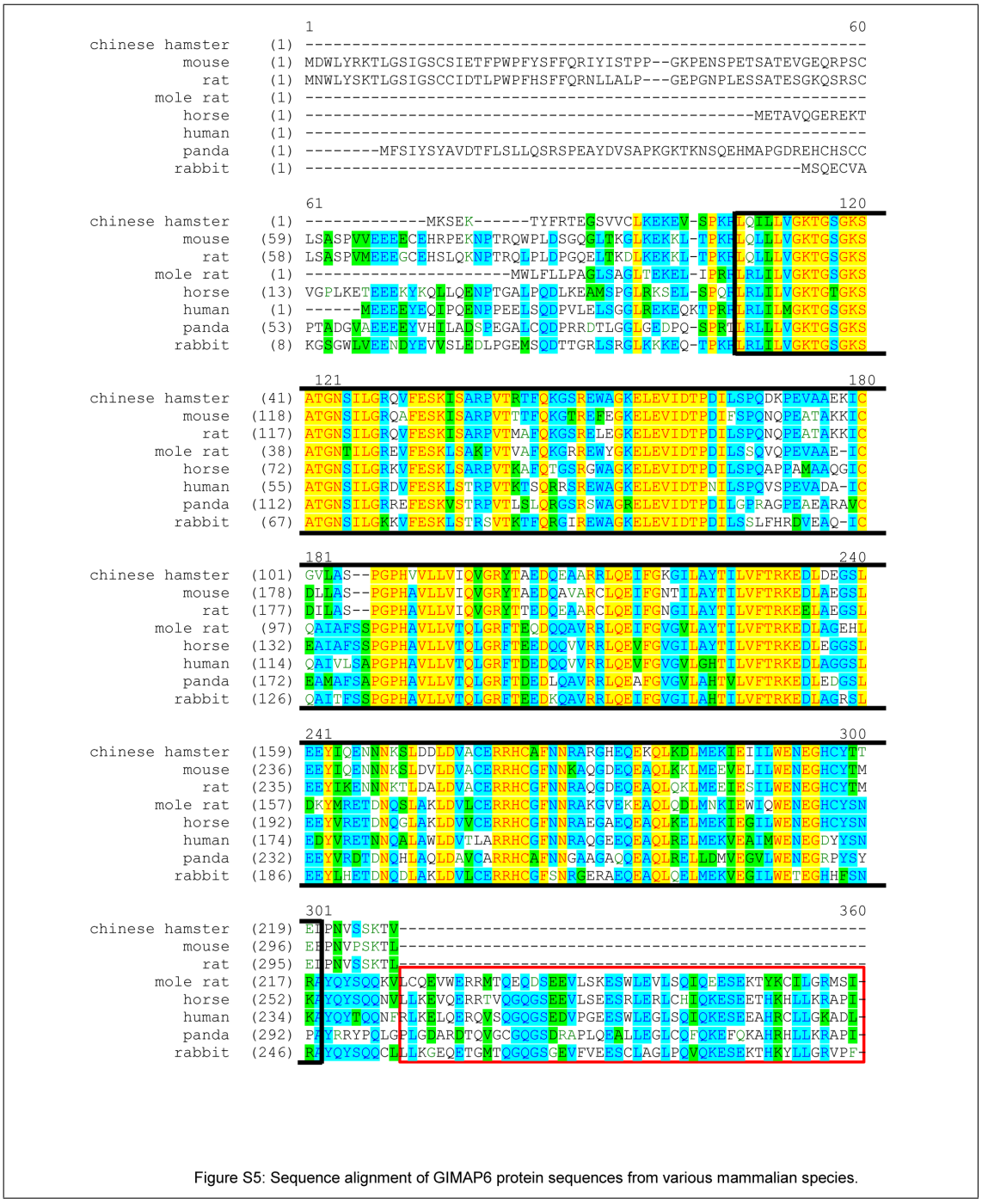

Figure 3.3.13: Sequence alignment of GIMAP6 protein sequences from various mammalian species.

Protein sequences were either taken directly from the NCBI protein database or were deduced from expressed DNA sequence tags or genomic sequences. The conserved AIG1/GTPase domain is boxed in black. The extended C-terminal region that is present in most mammals but absent from mouse, rat and chinese hamster, is boxed in red. Figure taken from Pascall et al. 2013. One-hundred percent type-conserved amino acid residues are shown in red, while at least 60% type-conserved residues are in blue.

The C-terminal domains within these organisms are truncated due to alternative splicing; however, this difference in splicing does not affect encoding of the GTPase/AIG1 domain. As part of a routine mapping exercise, GIMAP6 variants (human) with the N-terminal region (amino acids 1-36) and C-terminal region (amino acids 243-292) truncated were assayed to check whether they could bind GABARAPL2. As mentioned earlier the N-terminal region contains within it a putative AIM that does not appear to be involved in the interaction (Figure 3.3.3). Deleting the N-terminal region had no effect on the interaction with GABARAPL2 (Figure 3.3.14). Truncating the C-terminal region of GIMAP6, however, did abrogate the interact with GABARAPL2. The C-terminal domain of hGIMAP6 (amino acids 243-292) is predicted to contain two α-helical extensions (Figure 6.3.1) and given that α-helices are often found at the interface of protein-protein interaction these may be involved in the interaction with GABARAPL2. Constructs were prepared such that hGIMAP6 was truncated in its C-terminal region in increments of 10 amino acids, up to the 50 terminal residues. Truncating the protein in these ways completely abrogated the interaction between GIMAP6 and GABARAPL2: i.e. deletion of the 10 terminal amino acid residues was alone sufficient to abolish the interaction (Figure 3.3.14).

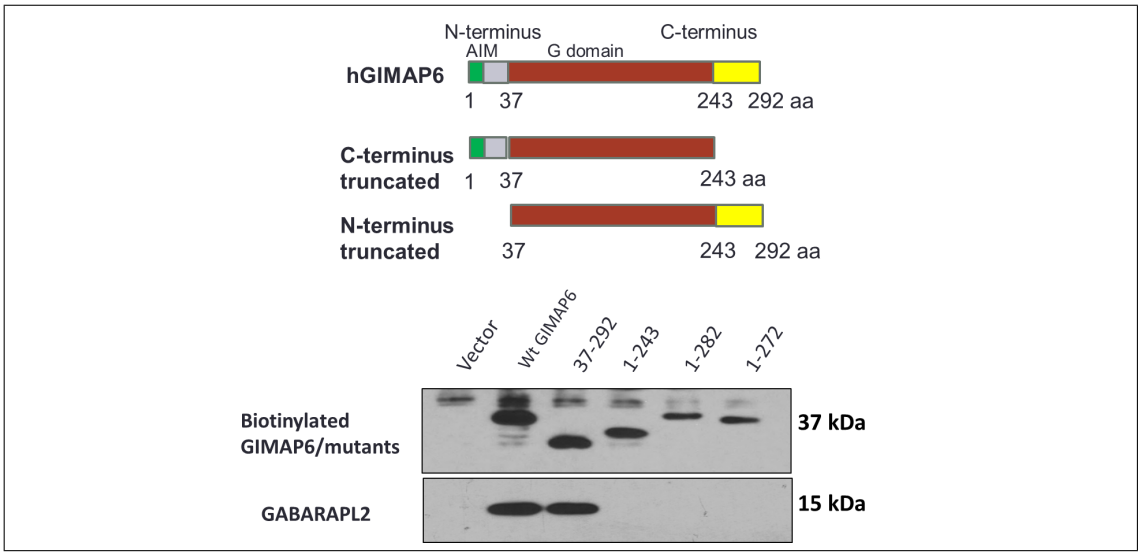

Figure 3.3.14: The C-terminus of GIMAP6 is required for the GIMAP6-GABARAPL2 interaction.
Upper panel; schematic diagram of truncations introduced within GIMAP6. Lower panel; HEK293T cells were transfected with 20 µg wt GIMAP6 or the indicated mutated derivatives in plasmid pcDNA3Biot1His6iresBirA. Biotinylated and associated proteins were recovered by streptavidin-agarose affinity purification 48h post transfection. Immunoblots of the recovered proteins were probed with the streptavidin-HRP conjugate (to show the GIMAP6 proteins; top panel of immunoblot) or rat monoclonal antibody MAC446 to GABARAPL2 followed by an HRP-conjugated goat F(ab')$_2$ fragment anti-rat IgG (bottom panel of immunoblot) to demonstrate co-purification of GABARAPL2. Truncation of the C-terminus but not the N-terminus abolishes GIMAP6-GABARAPL2 interaction.

This 10 amino acid sequence contains a number of charged residues (for e.g., histidine, arginine, lysine) that may play a role in the interaction with GABARAPL2 and, significantly, does not have any obvious sequence homology to an AIM sequence. Mutations were introduced within this C-terminal region of hGIMAP6 to pinpoint specific residues that may play a role in the interaction. The terminal 5 amino acids were removed, selected charged residues were mutated to alanine and the terminal leucine, that is completely conserved across all species in which GIMAP6 is expressed, was also mutated to alanine. The variant with the terminal 5 amino acid residues removed (GIMAP6$_{1\text{-}287}$) was able to interact with GABARAPL2, but at a noticeably reduced efficiency. This suggested that residues both inside and outside this last 5-residue stretch are involved in the interaction.

Mutations were introduced within this region; however, both the K289A and L292A variants of GIMAP6 were able to interact with GABARAPL2 as efficiently as wt hGIMAP6 (Figure 3.3.15).

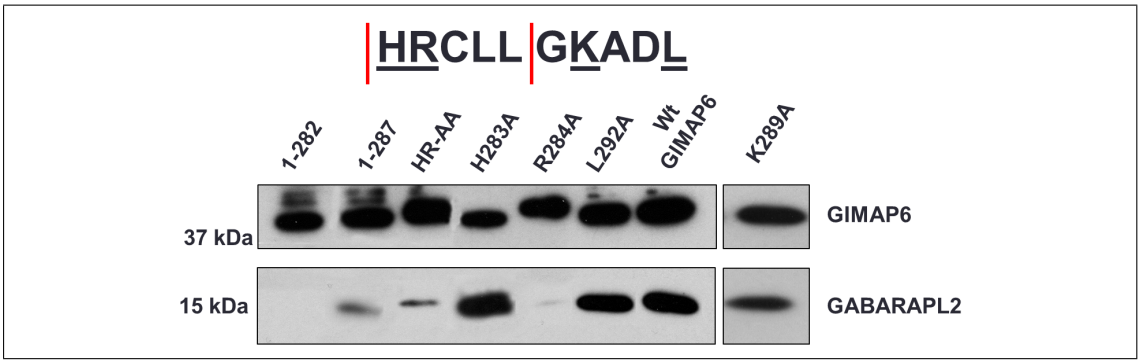

Figure 3.3.15: The ability of GIMAP6 variants with mutations within the terminal 10 amino acids to interact with GABARAPL2.
Upper panel: amino acid sequence of the residues in the C-terminal region of GIMAP6. Residues underlined were substituted with alanine. Red bars indicate the introduction of stop codons. Lower panel: HEK293T cells were transfected with 20 µg wt GIMAP6 or the indicated mutated derivatives in plasmid pcDNA3Biot1His6iresBirA. Biotinylated and associated proteins were recovered by streptavidin-agarose affinity purification 48h post transfection. Immunoblots of the recovered proteins were probed with the streptavidin-HRP conjugate (to show the GIMAP6 proteins: top panel of immunoblot) or rat monoclonal antibody MAC446 to GABARAPL2 followed by an HRP-conjugated goat $F(ab')_2$ fragment anti-rat IgG (bottom panel of immunoblot) to demonstrate co-precipitation of GABARAPL2.

The lysine residue was predicted to have a role in the interaction due to its charged nature, but pull-down experiments showed that the K289A variant was able to interact with GABARAPL2 in a manner similar to that of the interaction between wt GIMAP6 and GABARAPL2. Mutations were then introduced within resides spanning amino acid positions 283-287 of hGIMAP6. While mutating the histidine at position 283 did not appear to affect the interaction, interestingly, the R284A variant exhibited poor interaction with GABARAPL2, as did the HR283,284AA variant (HR-AA). The results from these interaction studies indicate that the arginine at position 284 is important for the interaction, as are residues within the terminal pentapeptide, and also indicate that the cumulative character of the terminal 10 amino acid residues within hGIMAP6 is critical

for the interaction with GABARAPL2.

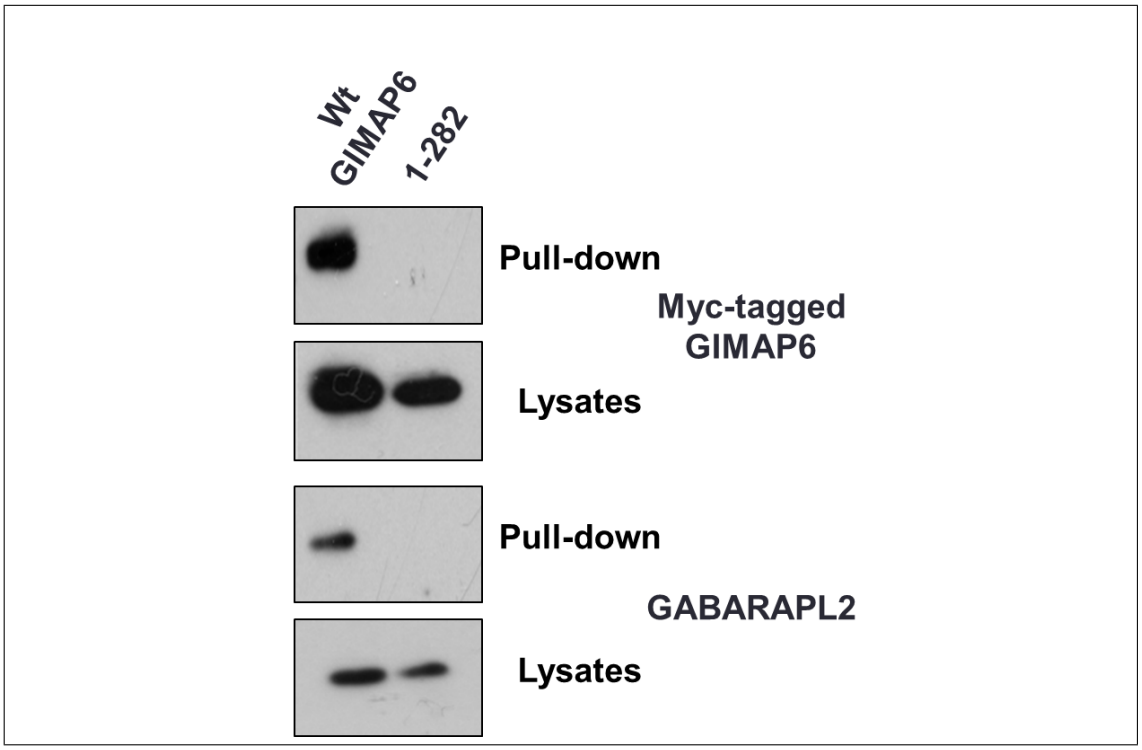

Figure 3.3.16: The GIMAP6$_{1\text{-}282}$ variant is unable to bind GTP-agarose.
Cell lysates were prepared from HEK293 cells that had been engineered to stably express either myc-tagged wt GIMAP6 or myc-tagged GIMAP6$_{1\text{-}282}$. Lysates were incubated with GTP-agarose beads overnight at 4 ºC. Bound proteins were eluted off the beads by boiling and proteins separated by SDS-PAGE. Separated proteins were analysed by Western blotting using either MAC446 followed by an HRP-conjugated goat F(ab')$_2$ fragment anti-rat IgG (bottom panel of immunoblot) to demonstrate co-purification of GABARAPL2 (right blot, lower half), or anti-myc monoclonal antibody 9E10 followed by HRP-conjugated goat anti-mouse IgG to detect the myc-tagged GIMAP6 and the variant. As expected wt GIMAP6 can bind GTP-agarose and can co-purify GABARAPL2. The variant cannot bind GTP-agarose.

The interaction between hGIMAP6 and GABARAPL2 could be a result of an interaction between terminal helices present within both proteins: the structure of GABARAPL2 has been solved to show the presence of two α-helical extensions within its N-terminal domain (Paz et al., 2000). Alternatively, the C-terminal region of hGIMAP6 may play a role in maintaining the overall structural integrity of the molecule. wt hGIMAP6 has been shown to interact with GDP and GTP (see (subsection 3.3.4)). I carried out experi-

ments to investigate whether the C-terminally truncated variant, GIMAP6$_{1\text{-}282}$, was able to bind GTP using lysates from stable cell lines expressing myc-tagged wt GIMAP6 and myc-tagged GIMAP6$_{1\text{-}282}$ for GTP-agarose affinity purifications. While wt GIMAP6 was able to bind GTP, the GIMAP6$_{1\text{-}282}$ variant was not, thus indicating that this region may play a structural role in GTP binding by GIMAP6, and thereby in its interaction with GABARAPL2. GABARAPL2 could be co-precipitated with GIMAP6 via GTP-agarose affinity, but could not with the variant, as expected (Figure 3.3.16).

3.3.6 A GIMAP6 variant that is unable to interact with GABARAPL2 does not re-locate to autophagosomes on induction of autophagy

GIMAP6 has been shown to translocate to autophagosomes on the induction of autophagy, where it co-localises with the classic autophagosomal marker MAP1LC3B, and GABARAPL2 (Pascall et al., 2013). This phenomenon is also observed in Jurkat T cells and in primary Human Umbilical Vein Endothelial Cells (HUVEC) in which GIMAP6 is endogenously expressed. Could the variant GIMAP6 protein that was unable to interact with GABARAPL2 also exhibit a similar translocation on induction of autophagy? Stable HEK293 cell lines expressing myc-tagged wt GIMAP6 and myc-tagged GIMAP6$_{1\text{-}282}$ were used to carry out these experiments. Cells were treated with starvation medium (amino acid starvation) for 90 min to induce autophagy. Cells were then fixed with paraformaldehyde and permeabilised using methanol. Following this, cells were processed for immunocytochemistry using MAC445 (anti-hGIMAP6) and rabbit anti-MAP1LC3B. In contrast to wt GIMAP6, the variant GIMAP6 was not recruited to the autophagosomes under starvation (autophagic) conditions (Figure 3.3.17). Expression of the variant GIMAP6 did not affect, in any obvious way, the re-localisation of MAP1LC3B (Figure 3.3.17) or GABARAPL2 to autophagosomes on induction of autophagy (not shown).

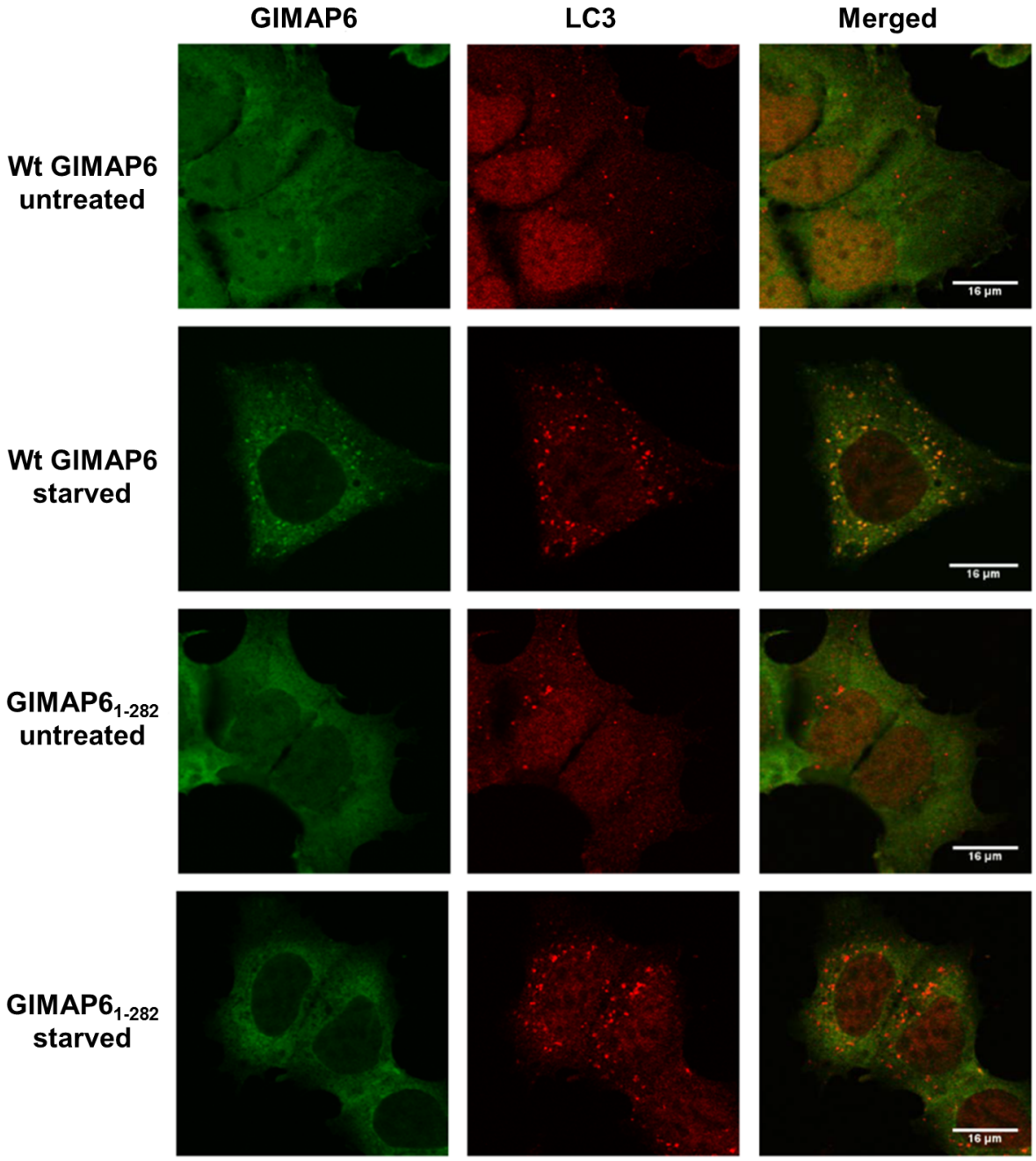

Figure 3.3.17: The C-terminal 10 amino acids of GIMAP6 are required for its recruitment to autophagosomes.
A stable HEK293 cell line expressing a myc-tagged GIMAP6 lacking the C-terminal 10 amino acids (GIMAP6$_{1-282}$) and the myc-wt GIMAP6 HEK293 cell line were either left untreated or were treated with starvation medium for 90 min to induce autophagy. Cells were fixed with 4% paraformaldehyde, permeabilised with methanol and then processed for immunocytochemistry using rat anti-human GIMAP6 monoclonal antibody MAC445 or rabbit anti-MAP1LC3B (Sigma) followed by an Alexafluor 488-conjugated anti-rat IgG or an Alexafluor 568-conjugated anti-rabbit IgG, respectively. Images were captured using an Olympus FV1000 imaging system. Scale bar represents 16µm.

This observation was also true for the myc-tagged GIMAP6$_{G50D}$ (Figure 3.3.18) and GIMAP6$_{S54N}$variants (not shown).

There could be a few possibilities for such an observation; (i) it is the interaction between GIMAP6 and GABARAPL2 that is responsible for the re-location of GIMAP6 to autophagosomes when autophagy is induced; (ii) truncating the C-terminal region of GIMAP6 affects its structure and consequently its GTP/GDP binding ability in such a manner that it prevents re-localisation to autophagosomes, or (iii) amino acids 283-292 of GIMAP6 interact with other proteins that mediate its recruitment to autophagosomes.

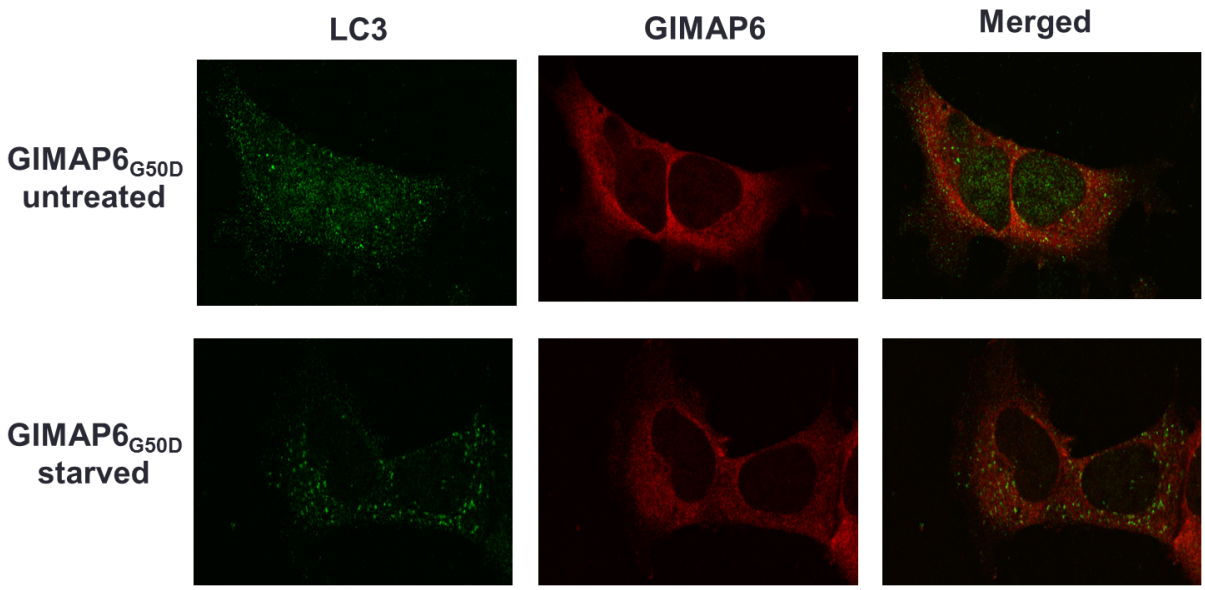

Figure 3.3.18: The GIMAP6$_{G50D}$ variant is unable to relocate to autophagosomes on induction of autophagy.
A stable HEK293 cell line expressing myc-tagged GIMAP6$_{G50D}$ was treated with starvation medium for 90 min to induce autophagy. Cells were fixed with 4% paraformaldehyde, permeabilised with methanol and then processed for immunocytochemistry using rat anti-human GIMAP6 monoclonal antibody MAC445 or rabbit anti-MAP1LC3B (Sigma) followed by an Alexafluor 568-conjugated anti-rat IgG or an Alexafluor 488-conjugated anti-rabbit IgG, respectively. Images were captured using an Olympus FV1000 imaging system.

3.4 Summary

In this chapter, I have shown, for the first time, that hGIMAP6 is able to bind GTP, and further showed that this region appears to be involved in the interaction of hGIMAP6 with GABARAPL2 (Table 3.4.1). The C-terminus of hGIMAP6, and in particular the terminal 10 amino acids are also required for the interaction with GTP and GABARAPL2. Earlier studies in our laboratory have shown that hGIMAP6 is able to relocate to autophagosomes on the induction of autophagy (amino acid starvation) (Pascall et al., 2013), and this relocation was abrogated when the ability of hGIMAP6 to bind GTP and GABARAPL2 was blocked.

Variant	Binds GTP	Binds GABARAPL2
wt hGIMAP6	Yes	Yes
$hGIMAP6_{Y6A}$	Unknown	Yes
$hGIMAP6_{I9A}$	Unknown	Yes
$hGIMAP6_{G50D}$	No	No
$hGIMAP6_{S54N}$	No	No
$hGIMAP6_{R134A}$	Yes	Yes
$hGIMAP_{1\text{-}282}$	No	No

Table 3.4.1: Summary of results.

4 Molecular requirements of GABARAPL2 for the interaction with GIMAP6

4.1 Context

GABARAPL2, also known as GATE-16 (Golgi-associated ATPase enhancer of 16 kDa), is a homologue of the yeast Atg8 protein. It is a ubiquitin-like protein that is thought to be involved in autophagosome biogenesis (Weidberg et al., 2010) and in the recruitment of cargo into autophagosomes, possibly via its interaction with NBR1 (Kirkin et al., 2009b,a), amongst other autophagy cargo receptors. While yeast possess one *Atg8* gene, mammalian cells express several Atg8-like proteins that, based on the similarities within their amino acid sequences, can be divided into two sub-families: the MAP1LC3 sub-group that contains the MAP1LC3A, MAP1LC3B, and MAP1LC3C proteins, and the GABARAP sub-group that contains GABARAP, GABARAP-like protein (GABARAPL) 1, GABARAPL2/GATE-16, and GABARAPL3. GABARAPL3 has been demonstrated to be expressed at only the transcriptional level and therefore is likely to be a pseudogene (Xin et al., 2001).

The importance of yeast Atg8 was demonstrated by a genetic screen carried out to detect mutants defective in autophagy (Tsukada and Ohsumi, 1993); the crucial nature of the

Atg8 proteins in the process of autophagy was further demonstrated by studies reviewed in (Shpilka et al., 2011). MAP1LC3B was the first mammalian Atg8 homologue to be identified (Kuznetsov and Gelfand, 1987), and remains the best studied, with its role in the process of autophagy now well established (Kabeya et al., 2000; Weidberg et al., 2010; Pankiv et al., 2007; Tanida et al., 2008; Lang et al., 1998; Scott et al., 1996; Weiergraber et al., 2013; Yang and Klionsky, 2009). GABARAPL2, on the other hand, has not been as well studied. GABARAPL2 was originally shown to be involved in intra-Golgi transport (Legesse-Miller et al., 1998). Further studies demonstrated that GABARAPL2 appeared to play a critical role in Golgi reassembly by interacting with NSF (N-ethylmaleimide sensitive factor), significantly enhancing its ATPase activity; the interaction with NSF in turn stimulated GABARAPL2's interaction with GOS-28, a Golgi v (vesicle)-SNARE (Soluble NSF attachment receptor) in an ATP dependent manner (Muller et al., 2002; Sagiv et al., 2000). The interaction between GABARAPL2 and GOS-28 was hypothesised to protect the v-SNARE and regulate SNARE function. That GABARAPL2 may have a role/s to play in the process of autophagy only became apparent with studies that reported the localisation of GABARAPL2 to autophagosomal membranes on the induction of autophagy, subsequent to its conjugation with phosphatidylethanolamine (PE) (Kabeya et al., 2004; Kirisako et al., 2000). A later study that attempted to dissect the exact role played by GABARAPL2 in autophagy reported that, unlike MAP1LC3B which was involved in the elongation of autophagosome membranes, GABARAPL2 mediated the process of autophagosome maturation (Weidberg et al., 2010).

4.1.1 The N-terminus of GABARAPL2

The crystal structure of GABARAPL2 demonstrated the presence of a ubiquitin-like fold with an N-terminal extension consisting of two α-helices (Paz et al., 2000). Given the significant similarities in amino acid sequence between members of the Atg8-like family, the three-dimensional fold observed in GABARAPL2 is seen in the other Atg8-like pro-

teins as well (Weiergräber et al., 2008; Rogov et al., 2013; Suzuki et al., 2014; Klionsky and Schulman, 2014; Paz et al., 2000; von Muhlinen et al., 2012). There may, however, be differences in conformational dynamics between the family members, particularly with respect to the N-terminal helical extensions. The first α-helix within MAP1LC3B contains residues with a positive charge, while the corresponding region within GABARAPL2 has a more hydrophobic character. While the N-terminal regions of both proteins can promote membrane tethering and fusion when they are cross-linked to liposomes (Weidberg et al., 2011), the differences between these regions may be why MAP1LC3B, but not GABARAPL2, is able to mediate the selective incorporation of SQSTM1 into autophagosomes (Shvets et al., 2011). The differences in the N-terminal region between MAP1LC3B and GABARAPL2 could also be why the two proteins may be involved in separate stages of autophagosome biogenesis (Weidberg et al., 2011, 2010). Does the N-terminal region of GABARAPL2 have a role to play in the interaction with GIMAP6? I have conducted experiments to attempt to answer this question.

4.1.2 The role of the AIM docking site within GABARAPL2 in the interaction with GIMAP6

As mentioned in subsection 3.1.2, the AIM can play a critical role in the interaction of binding partners with Atg8-like proteins. GIMAP6 has a peptide sequence consistent with a canonical AIM within its N-terminus. Introducing point mutations within this motif did not, however, affect interaction with GABARAPL2 (see Figure 3.3.3). The Atg8-related proteins bind to the AIM motif of a substrate, which has been shown to adapt an extended β-conformation, through their AIM docking site (ADS) (Rozenknop et al., 2011; Noda et al., 2008). The N-terminal helical domain of Atg8-related proteins consists of helices, α1 and α2; the presence of two helices within the N-terminal region that are attached to the convex face of the β-grasp fold is conserved amongst the Atg8 homologues (Weiergraber

et al., 2013; Paz et al., 2000).

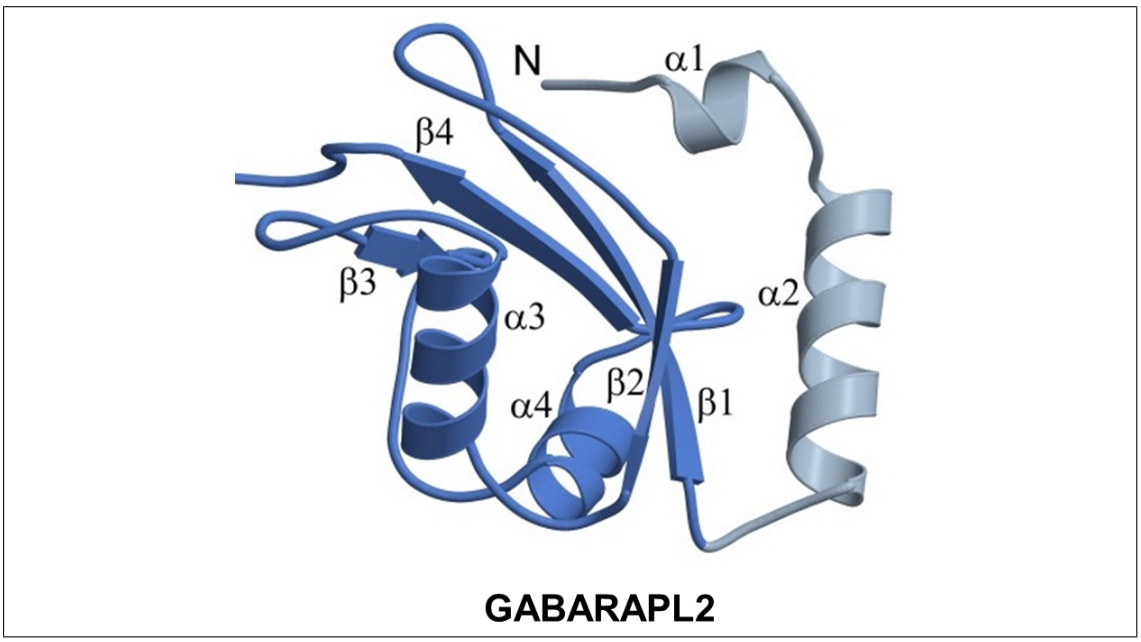

Figure 4.1.1: Representation of the fold of GABARAPL2.
The GABARAPL2 crystal structure (PDB ID 1EO6 Paz et al., 2000), contains the β-grasp motif (dark blue) which is a hallmark of the ubiquitin superfamily and is seen here decorated with by two N-terminal helical regions (light blue). Figure adapted from Weiergraber et al. (2013).

All Atg8-family proteins have an exposed β-strand (β2; corresponding to residues 48–52 of Atg8), which plays a role in the binding of the AIM through an intermolecular β-sheet. They also possess two characteristic hydrophobic pockets (hp1 and hp2; Figure 4.1.2) with hp1 comprising the side chains of Glu17, Ile21, Pro30, Ile32, Lys48, Leu50, and Phe104, while hp2 is composed of the side chains of Tyr49, Val51, Pro52, Leu55, Phe60, and Val63 (Noda et al., 2010, 2008; Behrends and Fulda, 2012). Residues within these pockets make contact with residues in the AIM and are required for AIM-mediated interactions. Does the ADS within GABARAPL2 play a role in the interaction with GIMAP6? I have attempted to answer this question.

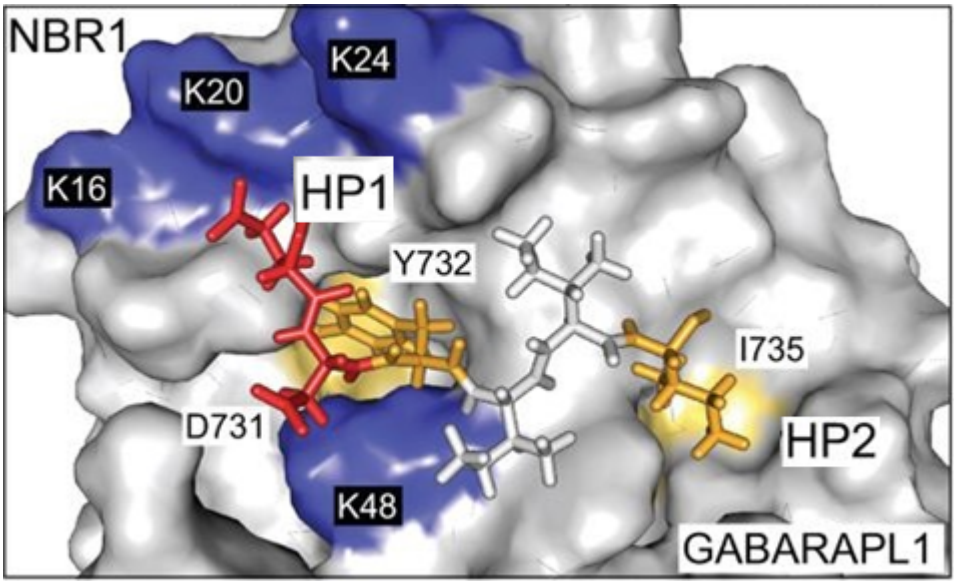

Figure 4.1.2: Representative example of the structural determinants of AIM-Atg8 interactions.
Surface representation of GABARAPL1 bound to the NBR1-LIR peptide. The hydrophobic pockets (hp1 and hp2) of GABARAPL1 are indicated in bright yellow. The amino acids (yellow) of the AIM peptide of GABARAPL1 that bind in the pockets are shown. Figure adapted from Birgisdottir et al. (2013).

4.1.3 Conjugation of phosphatidylethanolamine to GABARAPL2

Members of the Atg family of proteins play critical roles in autophagosome biogenesis (Xie et al., 2008). The Atg8-related family members are unique in that they can be conjugated to the lipid PE via a process that is analogous to protein ubiquitination. The conjugation of PE to Atg8 and the Atg8-related proteins has been shown to be required for their incorporation into the autophagosomal membrane (Ichimura et al., 2004, 2000). Investigations into the potential post-translational modifications of the Atg8-related family members were undertaken due to the detection of two species in immunoblot analysis of MAP1LC3B, with the two species corresponding to masses of 25 kDa and 23 kDa being termed MAP1LC3B-I and MAP1LC3B-II, respectively. Further investigation demonstrated the translation of MAP1LC3B as a full-length precursor (proMAP1LC3B; 30kDa).

This is rapidly cleaved near its C-terminal end by the cysteine protease Atg4 to allow the generation of MAP1LC3B-I and MAP1LC3B-II; MAP1LC3B-II is directly derived from MAP1LC3B-I via the conjugation to PE and its formation can be abrogated by mutating G120 to A (Fujita et al., 2008; Kabeya et al., 2000; Kirisako et al., 2000). To date four mammalian homologues of Atg4 have been reported: autophagin 1 (Atg4B), autophagin 2 (Atg4A), autophagin 3 (Atg4C), and autophagin 4 (Atg4D) with Atg4B likely to be the principal mammalian homologue (Mariño et al., 2003; Kirisako et al., 2000). Atg4B has been shown to cleave all members of the Atg8-related family of proteins (Hemelaar et al., 2003; Li et al., 2011), while Atg4A is specific for the GABARAP subfamily but not the MAP1LC3 subfamily (Scherz-Shouval et al., 2003; Li et al., 2011).

Members of the Atg8-related proteins are subsequently delipidated by the activity of Atg4 homologues (Kirisako et al., 2000), with a recent study suggesting that this delipidating activity of Atg4 is necessary for the recycling of lipidated members of the Atg8 family of proteins that are generated in a constitutive and non-selective manner on inappropriate membranes (Nakatogawa et al., 2012). The delipidating activity of the Atg4 homologues, a step regulated by reactive oxygen species, is required for the maintenance of a cytosolic pool of unlipidated Atg8-related proteins that can participate in autophagosome biogenesis. The C-terminal cleavage activity of the Atg4 homologues is dependent on the presence of Arg68 within MAP1LC3B; this residue forms a salt bridge with the Atg4 homologues (R65 within GABARAPL2) Liu et al. (2013), and the conserved glycine residue (G116 of GABARAPL2; G120 within MAP1LC3B); the protein is cleaved after this glycine residue (Kabeya et al., 2004, 2000). Mutating either residue to an alanine has been shown to abrogate Atg4 cleavage activity and consequently the lipidation of the MAP1LC3B (Liu et al., 2013; Kabeya et al., 2004). The cleaved Atg8-like proteins with the exposed glycine serve as a substrate for the ubiquitin-like conjugation reaction carried out by the E1-like enzyme Atg7, the E2-like enzyme Atg3, and the E3-like enzyme, the Atg12-Atg5-Atg16 complex, resulting in the covalent conjugation of PE (Ichimura et al.,

2004; Mizushima et al., 1998; Tanida et al., 1999, 2002, 2001; Ichimura et al., 2000; Taherbhoy et al., 2011), thereby allowing the anchoring of the Atg8-like proteins to the autophagosomal membrane.

While GABARAPL2, like MAP1LC3B, can also be cleaved by Atg4, the presence of the lipidated form of GABARAPL2 is not always evident (subsection 3.3.2; Tanida et al., 2004). Further, there is a divergence in the tissue distribution of MAP1LC3B and GABARAPL2 hinting at the possibility of GABARAPL2's role in autophagy being tissue-dependent (Elazar et al., 2003). It may also be that in addition to autophagy, GABARAPL2 has other tissue-specific functions. In this study I have investigated whether G116 of GABARAPL2 is required for the interaction with GIMAP6. I have also conducted an experiment that attempts to confirm whether the Gly residue within GABARAPL2 is exposed to behave as a substrate for possible lipidation.

4.2 Chapter Aims

1. Identify domains within GABARAPL2 that are required for the interaction with GIMAP6
2. Investigate whether the putative ADS within GABARAPL2 plays a role in the interaction with GIMAP6
3. Carry out studies to investigate the role of the conserved Gly at position 116 within GABARAPL2 in the interaction with GIMAP6

4.3 Results

4.3.1 The role of the N-terminal region within GABARAPL2 in the interaction with GIMAP6

While the three-dimensional fold of the different Atg8 homologues is believed to be similar, the conformational dynamics may differ, with the N-terminal region of the Atg8 homologues, in particular, being capable of attaining alternative conformations (Weiergraber et al., 2013; Paz et al., 2000). As mentioned above (subsection 4.1.1), the first α-helix within GABARAPL2 has a more hydrophobic character than the corresponding region within MAP1LC3B, which contains a number of positively charged residues. To analyse the importance of the N-terminal helices of GABARAPL2 in the interaction with GIMAP6, constructs were prepared such that both α-helices within the N-terminal region of GABARAPL2 were truncated. The mutant GABARAPL2 sequences were generated via PCR mutagenesis and were cloned into the Biot1-His6-IRES-BirA plasmid. HEK293T cells were co-transfected with these constructs and with a plasmid encoding N-terminally myc-tagged GIMAP6. Lysates were prepared 48h post transfection, streptavidin affinity purification was performed and the recovered biotinylated and associated proteins were detected by western blotting using the streptavidin-HRP conjugate and the anti-myc mAb 9E10 (Figure 4.3.1).

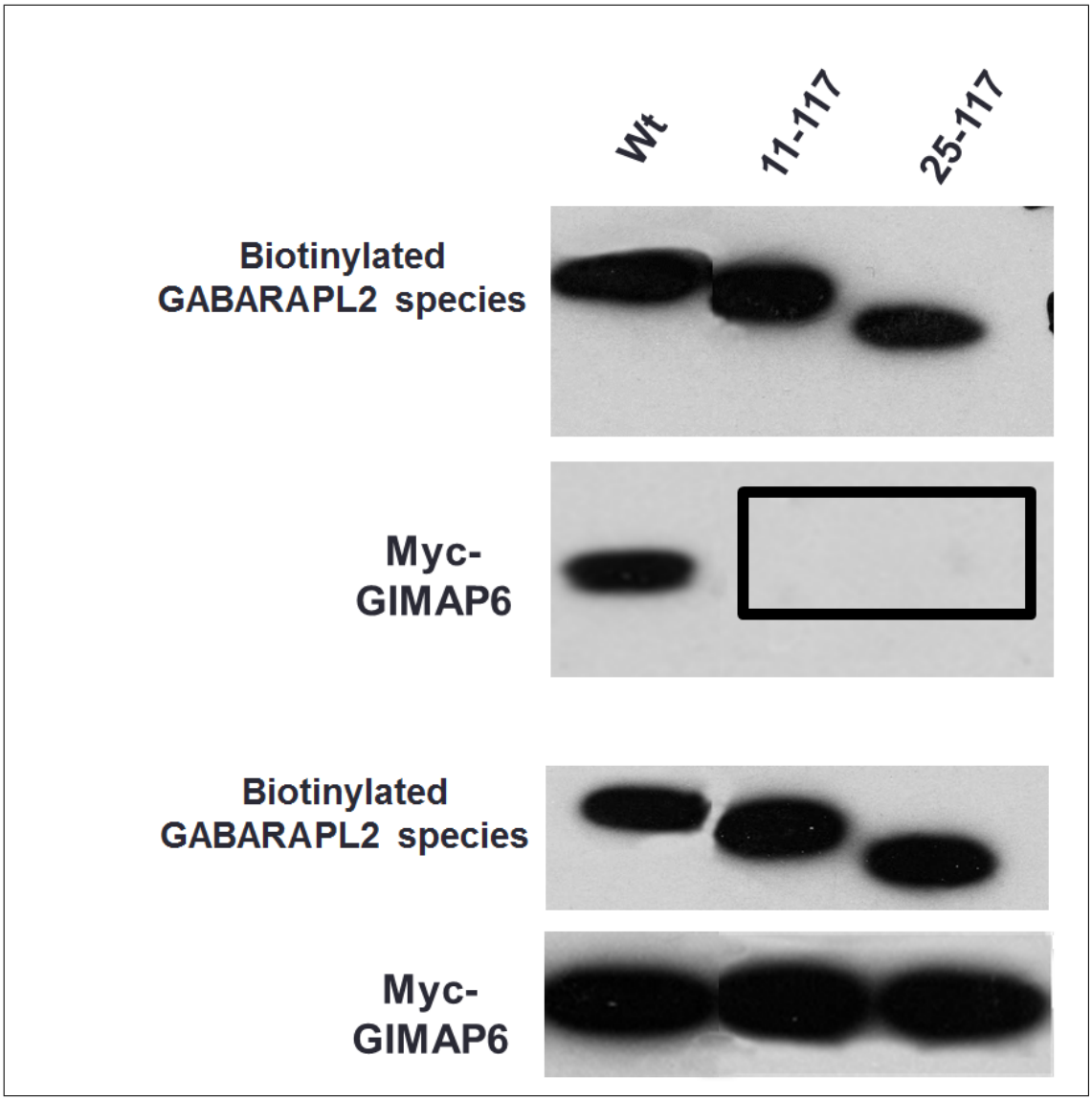

Figure 4.3.1: The N-terminal region of GABARAPL2 is required for the interaction with GIMAP6.

HEK293T cells were transfected with a plasmid encoding myc-GIMAP6 together with a second plasmid encoding GABARAPL2 or one of the two GABARAPL2 variants (all biotinylated), as indicated. Cell lysates were prepared and biotinylated and associated proteins recovered by streptavidin-agarose affinity chromatography. Western blots were probed with either the streptavidin-HRP conjugate to detect the biotinylated GABARAPL2 proteins or the anti-myc monoclonal antibody 9E10 followed by an HRP-conjugated goat anti-mouse IgG to detect myc-tagged GIMAP6.

Truncating GABARAPL2 in these ways completely abrogated its interaction with GIMAP6:

indeed, deletion of the first α-helix (residues 1-10) was sufficient to abolish the interaction. Based on this finding, I hypothesised that the first α-helix within the N-terminal region of GABARAPL2 would be sufficient for the interaction with GIMAP6. I prepared a chimera in which the first α-helix of GABARAPL2 replaced the corresponding α-helix in MAP1LC3B. The constructs were N-terminally myc-tagged and transfected into HEK293T cells along with a plasmid encoding biotin-tagged GIMAP6 (Figure 4.3.2).

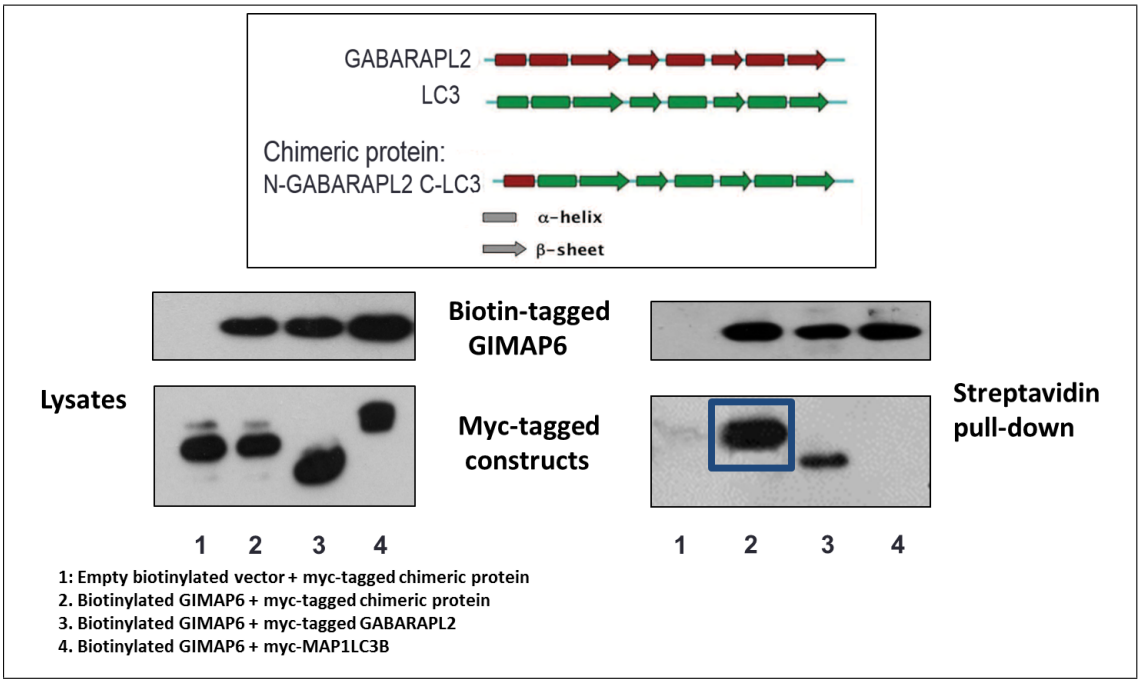

Figure 4.3.2: Swapping the first α-helix within MAP1LC3B with the corresponding helix found in GABARAPL2 results in a chimeric protein able to interact with GIMAP6.
Top panel: a schematic representation of the chimeric protein. Bottom panel: HEK293T cells were co-transfected with a plasmid encoding either myc-tagged GABARAPL2, myc-tagged MAP1LC3B, or the myc-tagged chimeric protein together with a plasmid encoding the biotinylated form of GIMAP6, as indicated. Cell lysates were prepared and biotinylated and associated proteins recovered by streptavidin-agarose affinity chromatography. Western blots were probed with either the streptavidin-HRP conjugate to detect the GIMAP6 protein or the anti-myc monoclonal antibody 9E10 followed by an HRP-conjugated goat anti-mouse IgG to detect the myc-tagged Atg8 homologues.

Lysates were prepared 48h post transfection and processed as described above. Interestingly, and as hypothesised, the chimeric protein with the N-terminus of GABARAPL2 swapped into MAP1LC3B was sufficient to bind GIMAP6, suggesting that this region

within GABARAPL2 is critical for the interaction.

4.3.2 The role of the ADS within GABARAPL2 in the interaction with GIMAP6

The ADS within Atg8 homologues plays a critical role in the interaction with a significant proportion of their binding partners (Behrends et al., 2010). As mentioned in subsection 4.1.2, residues within the hydrophobic pockets, hp1 and hp2, within the Atg8 homologues make contact with the residues from the AIM (particularly the tyrosine or the tryptophan at position 1 of the AIM, and the isoleucine or the leucine at position 4 of the AIM) and are critical for interactions that are dependent on the AIM (see Figure 4.1.2: the residues within hp1 and hp2 are conserved within GABARAPL1 and this figure is representative of structural determinants of AIM-ADS interactions). Here, constructs were designed to substitute the Tyr49 within hp2 and, separately, the Leu50 within hp1 of GABARAPL2 to Ala to investigate whether these residues have a role to play in the interaction of GABARAPL2 with GIMAP6. These residues were chosen for manipulation as a study characterising the interaction between the AIM of NBR1 with GABARAPL1 highlighted them (Rozenknop et al., 2011). They are completely conserved within the GABARAP sub-family, participate in the interaction with NBR1 (Rozenknop et al., 2011), and have been substituted in other studies investigating aspects of AIM-ADS interactions (Yamano et al., 2014; Shvets et al., 2011; Behrends et al., 2010). The mutant GABARAPL2 sequences were generated via PCR mutagenesis and were cloned into the Biot1-His6-IRES-BirA plasmid. HEK293T cells were co-transfected with this construct and with a plasmid encoding N-terminally myc-tagged GIMAP6. Lysates were prepared 48h post transfection, streptavidin affinity purification was performed and the recovered biotinylated and associated proteins were detected by western blotting using the streptavidin-HRP conjugate and the anti-myc mAb 9E10.

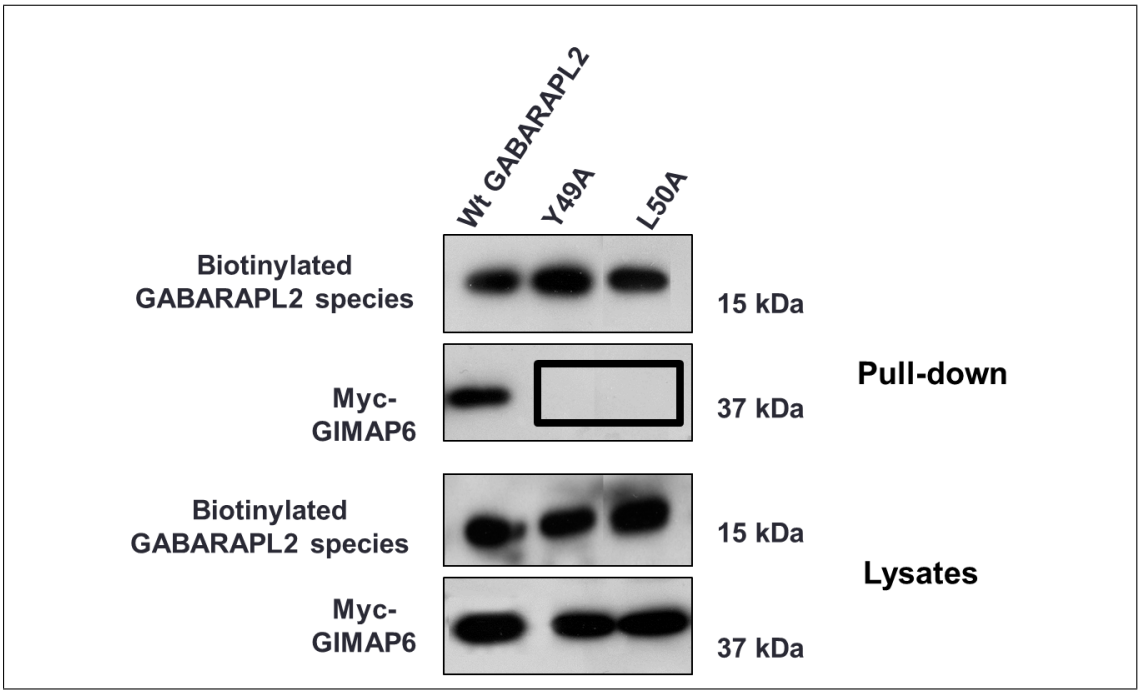

Figure 4.3.3: The ADS within GABARAPL2 plays an important role in the interaction with GIMAP6.
HEK293T cells were transfected with a plasmid encoding myc-GIMAP6 together with plasmids encoding biotinylated forms of GABARAPL2 or the GABARAPL2 variants as indicated. Cell lysates were prepared and biotinylated and associated proteins recovered by streptavidin-agarose affinity chromatography. Western blots were probed with either the streptavidin-HRP conjugate to detect the GABARAPL2 proteins or the anti-myc monoclonal antibody 9E10 followed by an HRP-conjugated goat anti-mouse IgG to detect myc-tagged GIMAP6.

Interestingly, as seen in Figure 4.3.3, substituting these residues in GABARAPL2 completely abrogated the interaction with GIMAP6. This finding hints at the possibility of the interaction between GABARAPL2 and GIMAP6 being mediated via an AIM. As discussed in the previous chapter (subsection 3.3.3), the N-terminal region of GIMAP6 contains a sequence that would be predicted to be part of an AIM but my experimental analysis argued against its relevance to the interaction under study. It may well be that there is a non-canonical, as yet unidentified AIM elsewhere within GIMAP6 that is required for the interaction with GABARAPL2. Indeed, non-canonical AIMs have recently been reported for proteins that interact with MAP1LC3C (von Muhlinen et al., 2012) and

for Atg12 (Kaufmann et al., 2014).

4.3.3 Role of the conserved glycine in GABARAPL2 in the interaction with GIMAP6

As introduced in subsection 4.1.3, the presence of the conserved glycine at the C-terminus of the Atg8 homologues is crucial for cleavage by members of the Atg4 family and their subsequent lipidation. Our research group has not been been able to detect the lipidation of GABARAPL2 on a consistent basis via western blot assuming, as the literature seems to suggest, that the PE-modified form of GABARAPL2 would display the same mobility shift as MAP1LC3B. Here, I have attempted to confirm that GABARAPL2 is cleaved after the conserved Gly116 which would potentially point to the possibility of it being lipidated at the glycine residue. This was done by engineering constructs that expressed wild-type GABARAPL2 and a variant of GABARAPL2 in which the Gly116 was substituted with an alanine. HEK293T cells were transfected with plasmids in which either wt GABARAPL2 or GABARAPL2$_{G116A}$was cloned into the pcDNA3 vector carrying a CMV promoter, an N-terminal biotinylation tag, a C-terminal myc tag (introduced via mutagenic PCR), and the internal ribosome entry site (IRES) sequence followed by *BirA* (Biot1-GABARAPL2-Myc-IRES-BirA). This plasmid was transfected into HEK293T cells and lysates were prepared 48h later. The transiently transfected HEK293T cells were also incubated in starvation medium for 90 min at 37 ºC. Lysates were separated via SDS-PAGE and transferred to a PVDF membrane and immunoblots were probed with either the streptavidin-HRP conjugate or the anti-myc mAb 9E10. The hypothesis was that if GABARAPL2 was normally cleaved by members of the Atg4 family, then no bands would be detected by the anti-myc mAb. Furthermore, if the Gly residue played a role in this cleavage then the GABARAPL2$_{G116A}$ mutant would not be cleaved at its C-terminal end and so would be detectable by the anti-myc ab. The results

were consistent with these propositions: wt GABARAPL2 was undetectable by the anti-myc mAb on western blotting, while GABARAPL2$_{G116A}$ was detected by the anti-myc ab (Figure 4.3.4). The reason behind the detection of certain protein bands (red asterisk Figure 4.3.4) is unclear. They are detectable by the anti myc mAb 9E10, but not by the streptavidin-HRP conjugate, indicating that they may correspond to degradation products that have lost their biotinylation tag but have maintained their C-terminal myc tag.

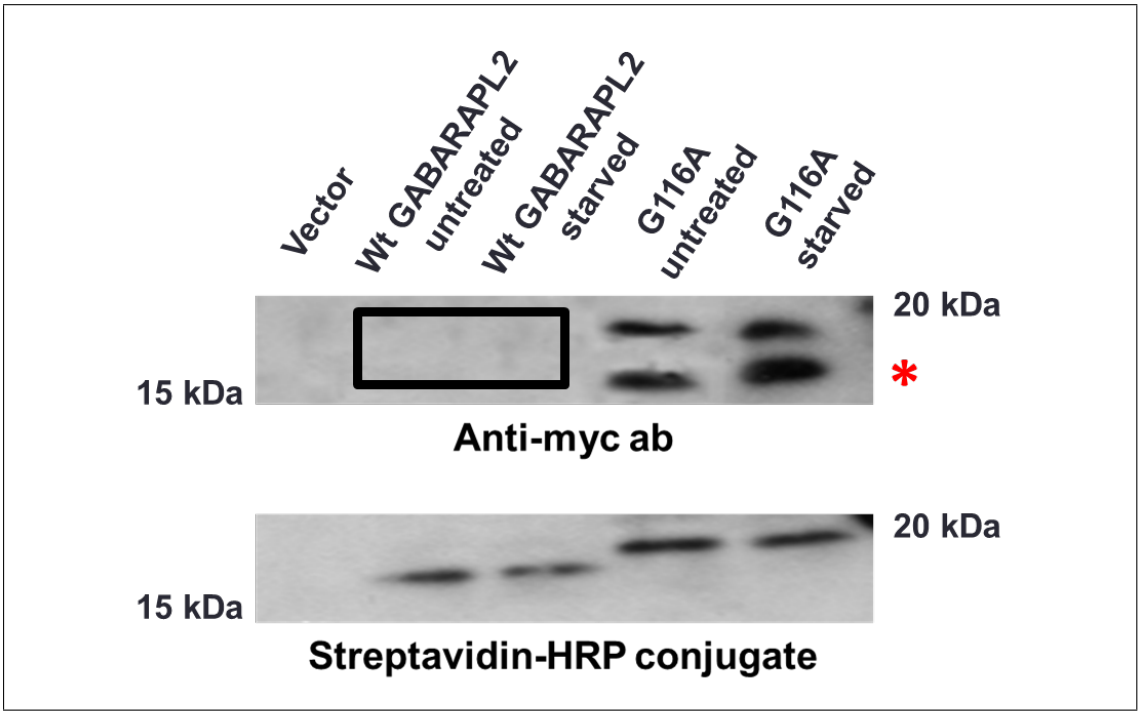

Figure 4.3.4: GABARAPL2 appears to be cleaved by Atg4.
HEK293T cells were transfected with a plasmid encoding GABARAPL2 or its G116A variant. Both forms carried an N-terminal biotinylation tag and were myc-tagged at the C-terminus. Cells were maintained in starvation medium at 37 ºC (or not) for 90 min approx. 48h post transfection. Following this, cell lysates were prepared and biotinylated and associated proteins recovered by streptavidin-agarose affinity chromatography. Western blots were probed with either the streptavidin-HRP conjugate or the anti-myc monoclonal antibody 9E10 followed by an HRP-conjugated goat anti-mouse IgG to detect the GABARAPL2 proteins. Red asterisk: possible degradation products.

The induction of autophagy had no obvious effect on the cleavage. Both wild-type and mutant GABARAPL2 were detected with the streptavidin-HRP conjugate with the wt GABARAPL2 migrating at a faster rate in the SDS-PAGE gel, providing further indica-

tion of its cleavage by Atg4.

Given that the conserved glycine residue of GABARAPL2 appears to be necessary for its potential lipidation and subsequent attachment to the autophagosomal membrane (the G116 mutant of GABARAPL2 appears to be unable to relocate to autophagosomes upon induction of autophagy: unpublished work from our group), I investigated whether G116 was required for the interaction between GIMAP6 and GABARAPL2. The mutant GABARAPL2 sequence was generated via PCR mutagenesis and was cloned into the Biot1-His6-IRES-BirA plasmid. HEK293T cells were transfected with this construct and, since they do not express GIMAP6 endogenously, they were also transfected with a plasmid encoding N-terminally myc-tagged GIMAP6. Lysates were prepared 48h post transfection, streptavidin affinity purification was performed and the recovered biotinylated and associated proteins were detected by western blotting using the streptavidin-HRP conjugate and the anti-myc ab.

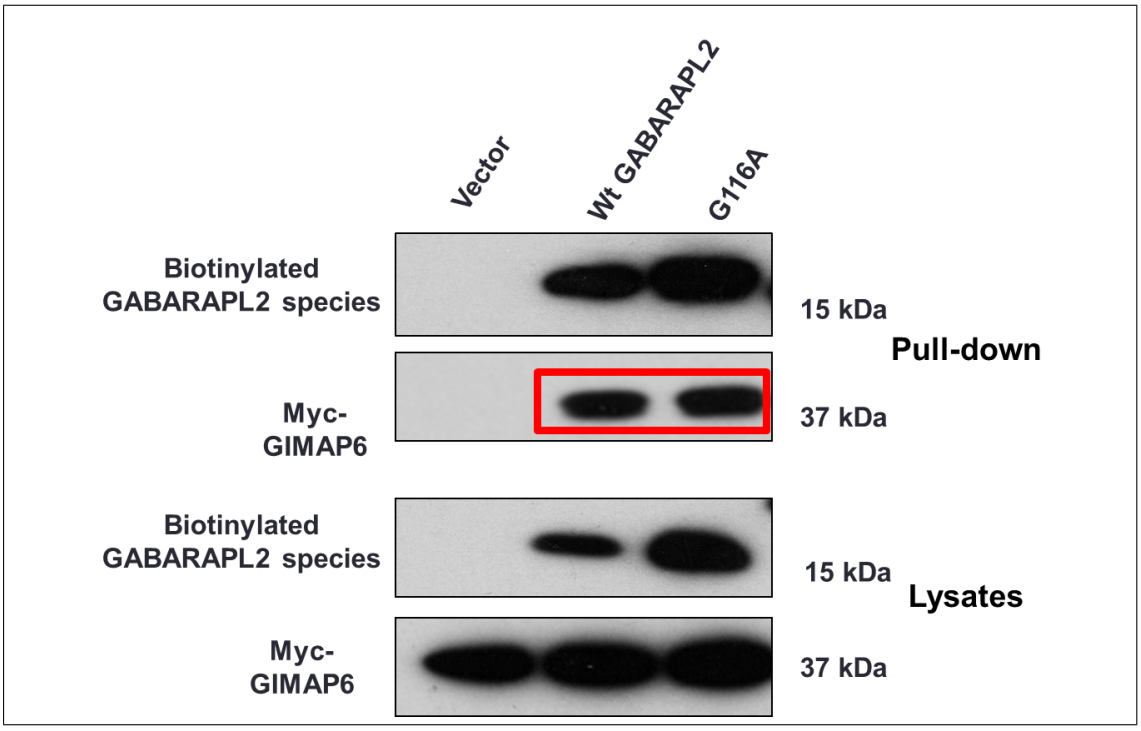

Figure 4.3.5: The conserved glycine116 within GABARAPL2 is not required for the interaction with GIMAP6.
HEK293T cells were transfected with a plasmid encoding myc-GIMAP6 together with plasmids encoding biotinylated forms of GABARAPL2, as indicated. Cell lysates were prepared and biotinylated and associated proteins recovered by streptavidin-agarose affinity chromatography. Western blots were probed with either the streptavidin-HRP conjugate to detect the GABARAPL2 proteins or an anti-myc monoclonal antibody 9E10 followed by an HRP-conjugated goat anti-mouse IgG to detect myc-tagged GIMAP6.

As seen in Figure 4.3.5, the G116A variant was successfully pulled down with GIMAP6. This indicates that G116 does not play a role in the interaction with GIMAP6. Hence, the interaction between these proteins could possibly occur in the cytosol prior to lipidation and the anchoring of GABARAPL2 to the autophagosomal membrane and so could be independent of the incorporation of GABARAPL2 into the autophagosome.

4.4 Summary

In this chapter, I have demonstrated that the N-terminus of GABARAPL2 plays an important role in the interaction with hGIMAP6. Interestingly, I also observed that the putative ADS within GABARAPL2 may have an important role to play in the interaction. Intriguingly, hGIMAP6 appears to be able to bind GABARAPL2 irrespective of its ability to be lipidated. Finally, while MAP1LC3B is unable to bind hGIMAP6, replacing the first α-helix within MAP1LC3B with the corresponding α-helix within GABARAPL2 rescued the interaction with hGIMAP6. These findings are further discussed in Chapter 6.

5 The GIMAP6-GABARAPL2 interaction is direct; GIMAP6 can also interact with GIMAP7

5.1 Chapter Aims

5.1.1 Is the GIMAP6-GABARAPL2 interaction direct?

GIMAP6 was shown to interact with GABARAPL2 in studies that were carried out with mammalian cell lysates. Due to the nature of these studies, it was not possible to comment on the status of this interaction, i.e. it could be either a direct interaction or one that requires additional proteins. Cross-linking studies hinted at the direct nature of the interaction between GIMAP6 and GABARAPL2 subsection 3.3.1. Given the M_r shifts observed but the relative inaccuracy of molecular weight estimations by SDS-PAGE meant that a requirement for additional, small proteins in a complex could not be excluded. Therefore I undertook *in vitro* studies with purified GIMAP6 and GABARAPL2 to investigate this issue.

5.1.2 Is the putative GIMAP6-GABARAPL2 complex detectable *in vivo*?

Given that GIMAP6 and GABARAPL2 could be detected as a complex upon cross-linking (subsection 3.3.1), are they detectable as a complex *in vivo*? This question was addressed by employing size exclusion chromatography using lysates obtained from either Jurkat T cells or from HEK293T cells that were transiently transfected with GIMAP6.

5.1.3 Can GIMAP6 interact with itself and can it interact with other members of the GIMAP family?

Recent structural studies carried out on members of the GIMAP family have shown that they can exhibit GTP-dependent oligomerisation, enabling scaffold formation e.g. on lipid droplets (GIMAP2; Schwefel et al., 2010), and can behave as GAPs, not only by displaying homodimerisation-stimulated GTP hydrolysis (GIMAP7), but also by stimulating GTP hydrolysis in a heterodimeric setting, i.e., GIMAP7 acting as a GAP for GIMAP2 (Schwefel et al., 2013). Can GIMAP6 interact with itself? Can it associate with other members of the GIMAP family? A pull-down approach was used to address these questions.

5.2 Results

5.2.1 The GIMAP6-GABARAPL2 interaction is direct

As mentioned above, the interaction between GIMAP6 and GABARAPL2 was detected in studies using mammalian cell lysates (Pascall et al., 2013). To address the issue of whether this interaction required additional proteins or was a direct interaction, *in*

vitro studies using purified proteins needed to be carried out. GST (control) and a GST-GIMAP6 fusion protein were expressed in *E.coli* (see subsection 3.3.4) and the two proteins column-purified on glutathione beads. The beads carrying the immobilised proteins were then incubated for 1 h at 4 ºC with 20 µg of purified GABARAPL2 that had been bacterially expressed (a kind gift from Michael Wilson, The Babraham Institute). The column was washed, the bound proteins (GST and GST-GIMAP6) were eluted from the beads with reduced glutathione and the eluates were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R to assess recovery of GST/GST-GIMAP6, and then western blotting was carried out to assay for the presence of GABARAPL2. Although GST was recovered at much higher levels than the GST-GIMAP6 fusion protein (technical issues concerning the purification of GIMAP6 have been described earlier in this thesis; subsection 3.3.4), GABARAPL2 eluted with GST-GIMAP6 but was not detected in the GST eluates. This indicated that the interaction between GIMAP6 and GABARAPL2 was direct and did not require other proteins (Figure 5.2.1).

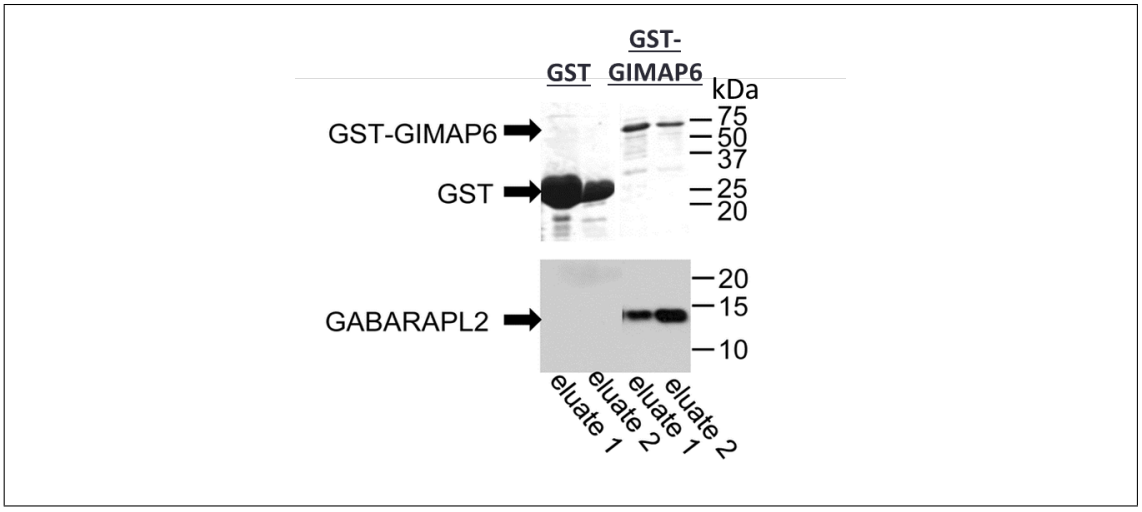

Figure 5.2.1: The interaction between GIMAP6 and GABARAPL2 is direct.
Glutathione Sepharose 4B-immobilised GST or GST-GIMAP6 was incubated with bacterially expressed purified GABARAPL2. Proteins were then eluted with glutathione and the eluates (two eluted fractions from each column), resolved by SDS-PAGE. Proteins were then visualised either by Coomassie Blue staining (top panel) or by Western blotting with anti-GABARAPL2 monoclonal antibody MAC446 followed by an HRP-conjugated goat F(ab')$_2$ fragment anti-rat IgG.

5.2.2 Existence of a GIMAP6-GABARAPL2 complex *in vivo*?

Studies in our group have demonstrated that GIMAP6 can regulate intracellular GABARAPL2 levels, i.e. an increase in the expression levels of GIMAP6 results in an increase in the level of GABARAPL2. This increase in protein levels was not observed for other members of the Atg8 family thus demonstrating a specificity for GABARAPL2. Interestingly, this regulation appears to be independent of any affect on *GABARAPL2* mRNA levels. Furthermore, a reduction in the endogenous levels of GIMAP6 resulted in reduced GABARAPL2 protein levels (Pascall et al., 2013). I asked whether this was a unidirectional effect (i.e. GIMAP6 levels affecting those of GABARAPL2) or whether the levels of the two proteins were mutually dependant. Experiments were carried out with Jurkat T cells engineered to express the tetracycline repressor protein (T-REx^{TM}) that were stably transfected with GABARAPL2 shRNA sequences. Cells were treated with either 1 μg/mL doxycycline for 4 days or maintained without doxycycline. A knockdown in the expression of GABARAPL2 was observed when the expression of the shRNA against GABARAPL2 was induced (Figure 5.2.2).

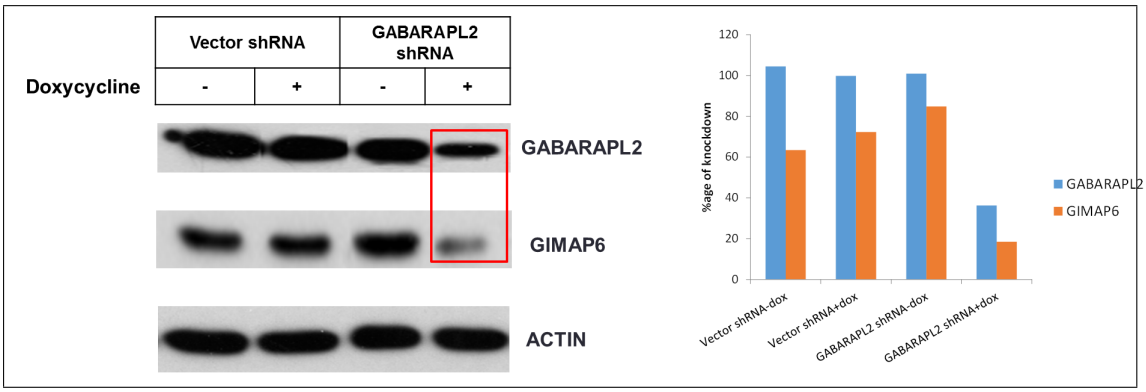

Figure 5.2.2: GABARAPL2 knockdown affects GIMAP6 protein levels.
Left panel: Jurkat T-REx^{TM}cells carrying GABARAPL2 shRNA sequences were either treated for 4 days with 1μg/mL doxycycline or were similarly maintained but in the absence of doxycycline. Cell lysates were prepared and assayed by western blotting for GIMAP6 (using mAb MAC445), GABARAPL2 (using mAb MAC446), or β-ACTIN followed by the appropriate HRP-conjugated secondary antibodies. Right panel: analysis of GABARAPL2/actin and GIMAP6/actin ratios.

Under these conditions, a downregulation in the levels of GIMAP6 was also observed, while β-actin levels remained unaffected. A possible explanation for this could be that GABARAPL2 is stabilised by the formation of a complex with GIMAP6, and vice versa. To test this hypothesis I decided to conduct size exclusion chromatography (SEC) experiments to investigate whether complexes of GIMAP6 and GABARAPL2 could be detected *in vivo* (*in cellulo*) (Figure 5.2.3). Size-exclusion chromatography is a widely used method for the estimation of protein molecular weight. It has the advantage of being non-denaturing and therefore permits the study of protein-protein interactions that occur normally *in vivo*. The widespread use and availability of fast-protein liquid chromatography (FPLC) systems enables the rapid generation and analysis of data.

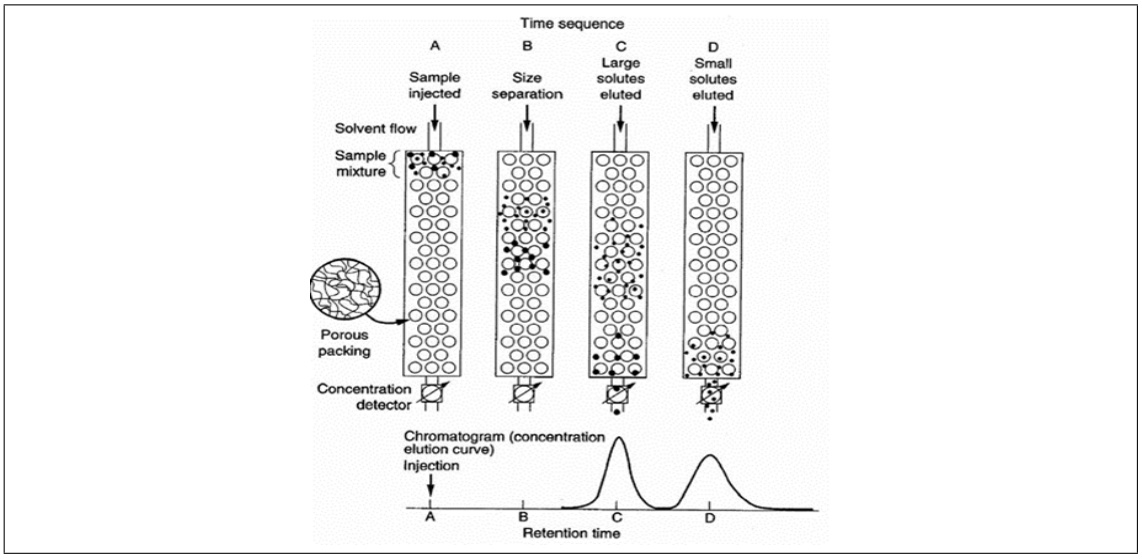

Figure 5.2.3: Schematic for Size Exclusion Chromatography.
A cartoon demonstrating the process of SEC. Larger molecules pass by the pores as they are too large to enter the pores, while smaller molecules get trapped. Hence, larger solutes elute before smaller ones.

Studies were carried out with lysates extracted from either the Biot-GIMAP6-His myc-BirA Jurkat T cell line, or from HEK293T cells transiently transfected with the Biot1-GIMAP6-His6-IRES-BirA plasmid. Following fractionation of the lysates on a Superdex-200 column, fractions were separated via SDS-PAGE and western blots were carried out

using the MAC445 and MAC446 antibodies against GIMAP6 and GABARAPL2, respectively. Jurkat T cells that over-expressed GIMAP6 were used with the idea of optimising the yield of stable complexes and increasing the likelihood of detecting a GIMAP6-GABARAPL2 complex *in vivo.* Biotinylated GIMAP6 has a molecular mass of approximately 40 kDa. Interestingly, however, its fractionation profile indicated that the majority of GIMAP6 eluted at volumes where proteins with molecular masses of $\geq$70-100 kDa would elute, with the peak in the profile indicating that GIMAP6 fractionated predominantly at a molecular mass of approximately 70 kDa. This could have been the result of homo/hetero GIMAP dimer formation, or could indicate the presence of a complex containing GIMAP6 and (an)other protein(s) - but not GABARAPL2, as GABARAPL2 did not appear to co-fractionate with GIMAP6 (Figure 5.2.4).

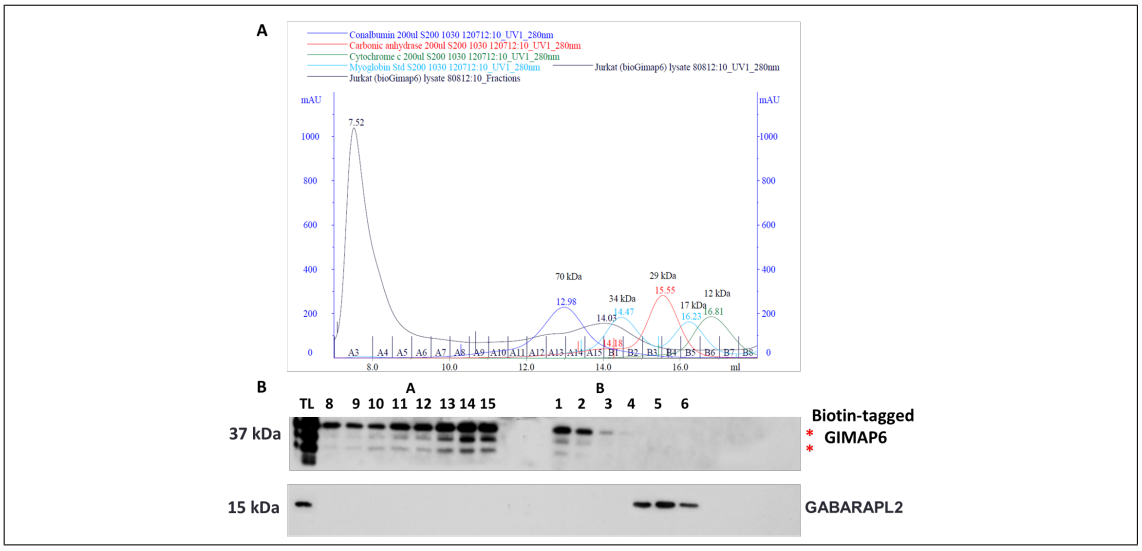

Figure 5.2.4: Gel filtration analysis of biotinylated GIMAP6 and endogenous GABARAPL2 expressed in Jurkat T cells.
A. Chromatogram representing analysis of gel filtration data via FPLC. Volumes at which various protein markers elute are shown. The grey line denotes the representative fractionation profile of lysates containing over-expressing biotinylated GIMAP6. A significant proportion of the lysate is eluted in the void volume. Individual fractions were collected and analysed via SDS-PAGE and western blotting. The x-axis denotes volumes at which proteins markers elute. The y-axis reports milli Absorbance units. **B**. Lysate extracted from Jurkat T cells stably expressing biotinylated GIMAP6. Lysate containing 0.5% NP-40 detergent was fractionated on a Superdex-200 gel filtration column. Individual fractions were collected and immunoblotted with MAC445 (upper panel) and MAC446 (lower panel) followed by an HRP-conjugated goat $(Fab')_2$fragment anti-rat IgG. Red asterisks denote possible GIMAP6 breakdown products. TL-total lysate.

The presence of GIMAP6 in the void volume fractions could indicate formation of oligomers containing GIMAP6. GABARAPL2, with a molecular mass of approximately 15 kDa, is reported to be able to form dimers (Paz et al., 2000) and eluted in fractions where a protein of approximately 15-30 kDa would be expected.

Could higher levels of GIMAP6 that may be obtained in a system where it is transiently expressed make it possible to detect the presence of a GIMAP6-GABARAPL2 complex *in vivo*? To test this, I used lysates extracted from HEK293T cells that had been transiently transfected with the Biot1-GIMAP6-His6-IRES-BirA plasmid to carry out further gel filtration experiments. Prior to this, I analysed the fractionation profile of

GABARAPL2 in HEK293T cells transiently transfected with the corresponding empty vector (Figure 5.2.5).

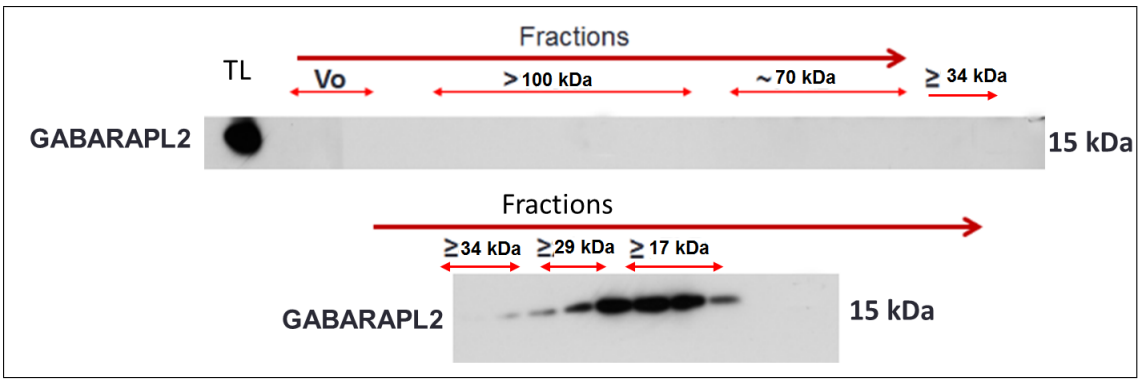

Figure 5.2.5: Gel filtration analysis of GABARAPL2 expressed in vector transfected HEK293T cells.
Lysate extracted from HEK293T cells 48h post transfection with control vector. Lysate containing 0.5% NP-40 detergent was fractionated on a Superdex-200 gel filtration column. Individual fractions were collected and immunoblotted with MAC446 followed by an HRP-conjugated goat $(Fab')_2$fragment anti-rat IgG. Red arrows with molecular masses above them denote the molecular masses of proteins that would be expected to elute at those volumes. TL- total lysate. V_o- void volume.

GABARAPL2 eluted at volumes where a protein of its size would be expected to fractionate, generating a fractionation profile similar to that seen using Jurkat T cells. Next, lysates from HEK293T cells that transiently over-expressed biotinylated GIMAP6 were analysed. Interestingly, while the fractionation profile of GIMAP6 was very similar to that seen in Jurkat T cells stably over-expressing GIMAP6, GABARAPL2 co-fractionated with GIMAP6, with no GABARAPL2 being detected in fractions where a protein of its size would normally elute (Figure 5.2.6).

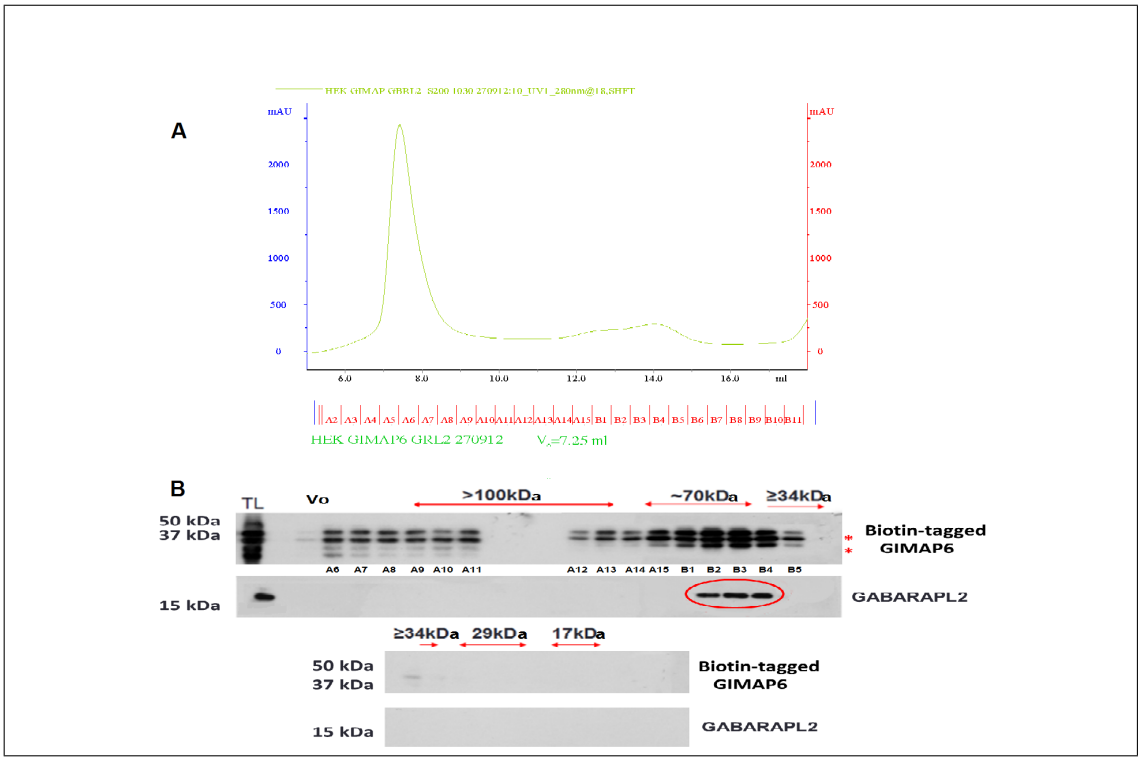

Figure 5.2.6: Gel filtration analysis of biotinylated GIMAP6 and endogenous GABARAPL2 and MAP1LC3B expressed in HEK293T cells.
A. Chromatogram representing analysis of gel filtration data via FPLC. The green line denotes the representative fractionation profile of lysates containing over-expressing biotinylated GIMAP6. A significant proportion of the lysate is eluted in the void volume. Individual fractions were collected and analysed via SDS-PAGE and western blotting. The x-axis denotes volumes at which proteins markers elute. The y-axis reports milli Absorbance units. **B.** Lysate extracted from HEK293T cells 48h post transfection with GIMAP6 in the pcDNA3Biot1His6iresBirA plasmid. Lysis buffer containing 0.5% NP-40 detergent was fractionated on a Superdex-200 gel filtration column. Individual fractions were collected and immunoblotted with either MAC445 or MAC446 followed by an HRP-conjugated goat $(Fab')_2$fragment anti-rat IgG. Red arrows with molecular masses above them denote the molecular masses of proteins that would be expected to elute at those volumes. TL- total lysate. Red asterisks indicate probable GIMAP6 breakdown products. Fractions in which GABARAPL2 co-fractionated with GIMAP6 are highlighted with the red circle.

This result indicated that GIMAP6 and GABARAPL2 could exist as a complex *in vivo*. The fractionation of GABARAPL2 coincided with the peak of the fractionation profile for GIMAP6 and eluted at volumes that would be in agreement with the molecular mass of a potential GIMAP-GABARAPL2 complex (approximately 55 kDa). In this experiment

the apparent co-migration of GIMAP6 and GABARAPL2 was supported by the internal control of the MAP1LC3B which migrated as a protein of its size would be expected to migrate (data not shown), thereby going some way towards eliminating the possibility of sample misidentification or gross denaturation. Unfortunately, the findings shown in Figure 5.2.6 were not reproduced in two further attempts. Another caveat that remains is that these experiments were done in HEK293T cells, and so whether this apparent co-migration could be replicated in Jurkat T cells remains to be seen. The reasons behind the lack of reproducibility are not understood. One possible source for this observation could be that the interaction is labile and easily disrupted when cell lysates are made. Inducing autophagy did not have any obvious effect on the fractionation profiles of GIMAP6 or GABARAPL2 (not shown).

5.2.3 GIMAP6 can interact with itself

As mentioned above, GIMAP6 eluted predominantly in gel filtration analysis where proteins with molecular masses of $\geq$70-100 kDa would elute, with the peak in the profile indicating that GIMAP6 fractionated predominantly at a molecular mass of approximately 70 kDa. This was observed in both Jurkat T cells, in which GIMAPs are endogenously expressed (experiments shown in this thesis were performed in Jurkat T cells stably over-expressing biotinylated hGIMAP6), and HEK293T cells, which exhibit no endogenous GIMAP expression. Given that hGIMAP6 runs at approximately 36 kDa and along with the recent evidence of the association between GIMAP2 and GIMAP7 (Schwefel et al., 2013), it was reasonable to hypothesise that possible reasons for the fractionation profile of GIMAP6 could be due to homodimerisation, heterodimerisation with another member of the GIMAP family (the molecular masses of members of this family, barring GIMAP8, are approximately 35 kDa) or, indeed, association with other proteins. Investigating whether GIMAP6 could interact with itself or with other members of the GIMAP family was feasible given the reagents already available in the laboratory and I performed

immunoprecipitation experiments to elucidate whether hGIMAP6 could interact with itself and/or other member/s of the hGIMAP family.

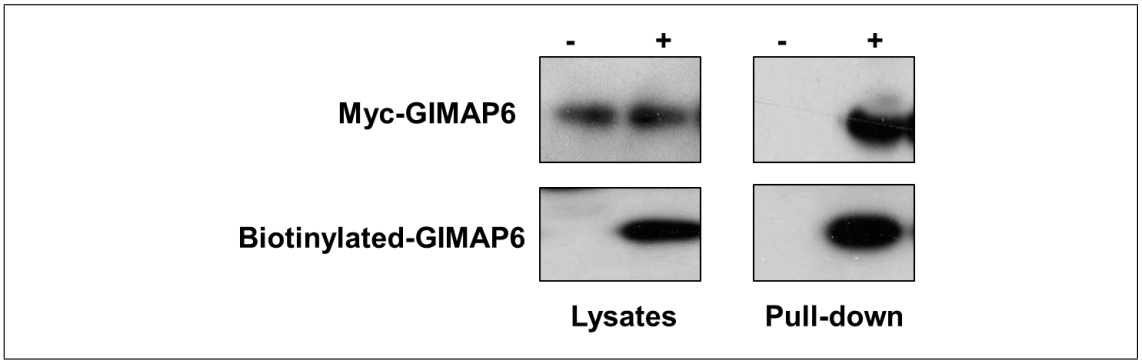

Figure 5.2.7: GIMAP6 can interact with itself.
HEK293T cells were transfected with a plasmid encoding myc-GIMAP6 together with a plasmid encoding biotinylated form of GIMAP6 (+) as indicated. (-) Cells transfected with empty vector and plasmid encoding myc-tagged hGIMAP6. Cell lysates were prepared and biotinylated and associated proteins recovered by streptavidin-agarose affinity chromatography. Western blots were probed with either the streptavidin-HRP conjugate to detect biotinylated GIMAP6 or the anti-myc monoclonal antibody 9E10 followed by an HRP-conjugated goat anti-mouse IgG to detect myc-tagged GIMAP6. -: Control biotinylation vector and myc-GIMAP6 co-transfected; +: Biotinylated GIMAP6 and myc-GIMAP6 co-transfected.

HEK293T cells were transiently co-transfected with plasmids encoding biotinylated GIMAP6, or the corresponding empty vector, and a plasmid encoding myc-tagged GIMAP6. Subsequently, lysates were prepared 48h post transfection. Streptavidin affinity purification was performed and the recovered biotinylated and associated proteins were detected by western blotting using the streptavidin-HRP conjugate and anti-myc mAb 9E10 (Figure 5.2.7). Myc-tagged GIMAP6 co-precipitated with biotinylated GIMAP6 indicating that GIMAP6 could interact with itself. Next, I attempted to investigate whether inserting mutations within GIMAP6 would affect its ability to interact with itself. I chose to use the following variants of GIMAP6: $GIMAP6_{S54N}$, a variant that affected the GTP binding ability of GIMAP6; $GIMAP6_{R134A}$, a variant hypothesised to affect the dimerisation of GIMAP6, $GIMAP6_{37\text{-}292}$, to investigate whether this variant that could bind GABARAPL2 could interact with itself, and $GIMAP6_{1\text{-}282}$,a variant unable to bind GTP

and GABARAPL2. HEK293T cells were co-transfected with biotinylated wt GIMAP6, or variants thereof, and with myc-tagged wt GIMAP6. The results in Figure 5.2.8 show that neither disrupting the termini of GIMAP6 nor mutating the arginine residue that abrogates the homodimerisation of GIMAP2 (Schwefel et al., 2010) appeared to disrupt the ability of biotinylated GIMAP6 (or relevant variants thereof) to pull-down myc-tagged GIMAP6.

Interestingly, the conserved arginine does not interfere with the dimerisation of GIMAP7, but does abrogate its GTPase activity (Schwefel et al., 2013). It remains to be seen what role this conserved residue has in GIMAP6. Both GIMAP2 and GIMAP7 have been shown to dimerise in a GTP-dependent manner and so I hypothesised that introducing a mutation within the GTPase domain of GIMAP6, thereby potentially inactivating it, might affect its ability to interact with itself. This was not observed. As seen in the figure, variants of GIMAP6 that were unable to interact with GABARAPL2 were able to pull-down wt myc-tagged GIMAP6 implying that the ability to dimerise or oligomerise is independent from the ability of GIMAP6 to interact with GABARAPL2. Similar results were obtained when mutant versions of both biotinylated and myc-tagged GIMAP6 were transiently co-transfected in HEK293T cells (not shown). Taken together these results imply that GIMAP6 has a tendency to oligomerise and does so in a manner that appears to be independent of a functioning GTPase domain. A number of GTPase families, including members of the dynamin superfamily, the septins (Sirajuddin et al., 2007), and the septin-related Toc proteins (Sun et al., 2002; Koenig et al., 2008) display GTPase activity that is triggered by the nucleotide-dependent dimerisation of their G domains (Gasper et al., 2009). Further studies are necessary to investigate the structural basis of the apparent oligomerisation of GIMAP6 and its role in its GTPase cycle and biological function.

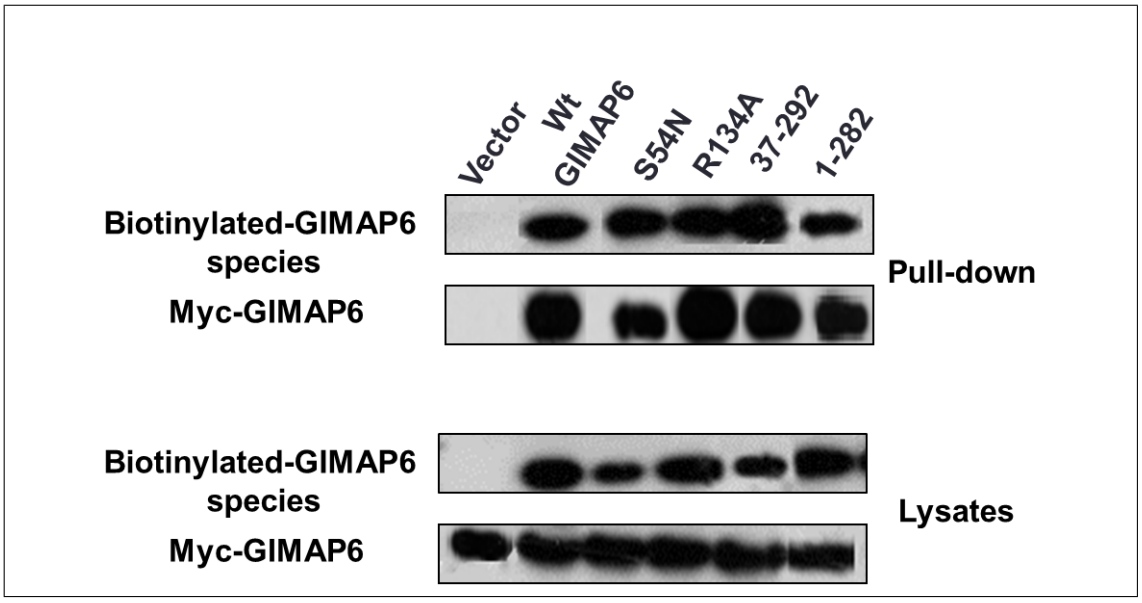

Figure 5.2.8: Selected mutations within GIMAP6 did not affect its ability to interact with itself.
HEK293T cells were transfected with a plasmid encoding myc-GIMAP6 together with plasmids encoding biotinylated forms of wt GIMAP6 or the biotinylated GIMAP6 variants as indicated. Cell lysates were prepared and biotinylated and associated proteins recovered by streptavidin-agarose affinity chromatography. Western blots were probed with either the streptavidin-HRP conjugate to detect biotinylated GIMAP6 or the anti-myc monoclonal antibody 9E10 followed by an HRP-conjugated goat anti-mouse IgG to detect myc-tagged GIMAP6.

5.2.4 GIMAP6 is able to interact with GIMAP7

In a number of GTPase families interactions between members of two functionally distinct subgroups have been shown to modulate catalytic activity. An example is the IRG. Members of one IRG subgroup assemble with proteins of the second subgroup to prevent GTP binding and activation (Hunn et al., 2008). Heterodimer formation and subsequent GTPase activation is also observed in the signal recognition particle (SRP) and its receptor (SRPR) (reviewed in Grudnik et al. 2009). Interestingly, recent research on the structure and on the nature of GTP binding and hydrolysis by members of the GIMAP family has demonstrated an interaction between GIMAP2 and GIMAP7 (Schwefel et al., 2013). Studies in the Ras superfamily of proteins have demonstrated that GTP hydrolysis

is stimulated by association with GAPs which often supply a catalytic arginine residue in *trans* to complement the active site (Bos et al., 2007b). Schwefel et al. (2013) have shown that a highly conserved arginine, corresponding to R134 in GIMAP6, from the conserved box motif in the GIMAP G-interface, has a dual function. While in the GIMAP2 homodimer, it stabilises the dimerisation interface (Schwefel et al., 2010), the equivalent arginine residue in GIMAP7 acts as a catalytic arginine finger in the GIMAP7 homodimer and GIMAP7-GIMAP2 heterodimer, by complementing the active site of the opposing monomer. Thus, the conserved box arginine serves a dual function by promoting self-association and stimulating GTP hydrolysis.

I attempted to investigate whether GIMAP6 could interact with other members of the GIMAP family using Jurkat T cells engineered to stably over-express biotinylated GIMAP6. Streptavidin affinity purification was carried out and the recovered biotinylated proteins and associated proteins were detected by western blotting using the streptavidin-HRP conjugate and mAbs against other members of the GIMAP family that had been generated previously in the laboratory. Interestingly, GIMAP7, but not other GIMAPs co-precipitated with biotinylated GIMAP6 (Figure 5.2.9)(A); the induction of autophagy did not appear to have any effect on the interaction (not shown).

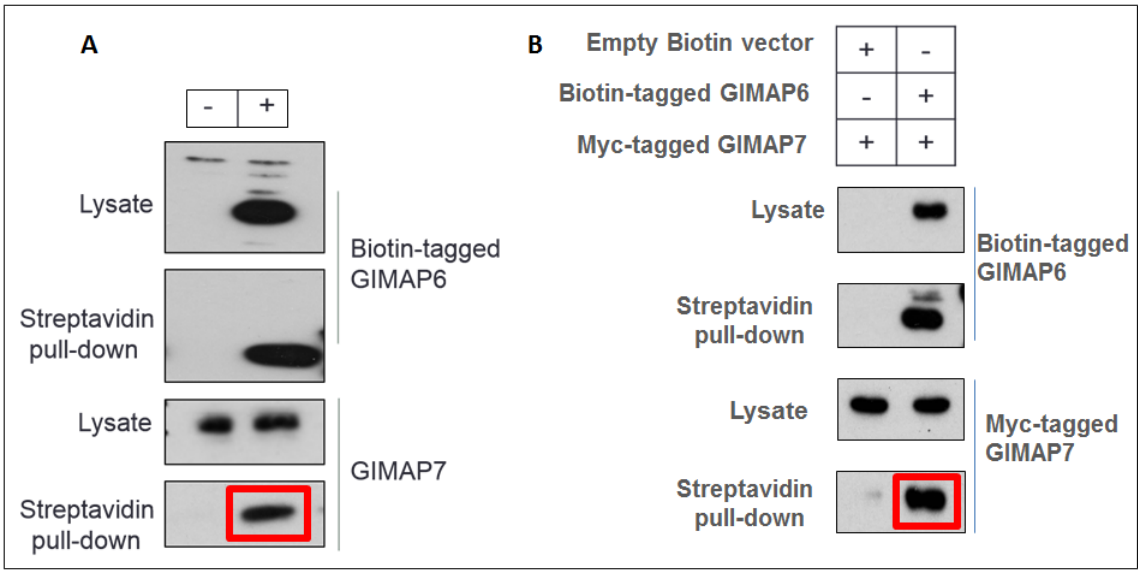

Figure 5.2.9: GIMAP7 is a binding partner for GIMAP6.
Left panel: Lysates were isolated from Jurkat T cells stably expressing either empty vector (-) or biotin-tagged GIMAP6 (+). Right panel: HEK293T cells were transfected with a plasmid encoding myc-GIMAP7 together with a plasmid encoding biotinylated wt GIMAP6 as indicated. In both cases, biotinylated and associated proteins were recovered by streptavidin-agarose affinity chromatography. Western blots were probed with the streptavidin-HRP conjugate to detect biotinylated GIMAP6. GIMAP7 expressed in the Jurkat T cells was detected with the rat monoclonal antibody to GIMAP7, MAC447, followed by an HRP-conjugated goat F(ab')$_2$ fragment anti-rat IgG. Myc-tagged GIMAP7 expressed in the HEK293T cells was detected by the anti-myc monoclonal antibody 9E10 followed by an HRP-conjugated goat anti-mouse IgG.

To follow up, HEK293T cells were transiently co-transfected with biotin-tagged GIMAP6 and myc-tagged GIMAP7 and pull-down experiments were performed. In confirmation of the earlier finding, GIMAP7 co-purified with biotinylated GIMAP6 (Figure 5.2.9)(B). Next, in order to determine the molecular requirements for the GIMAP6-GIMAP7 interaction, biotin-tagged GIMAP6 mutants were transiently co-transfected with myc-tagged wt GIMAP7. Interestingly, disrupting the N-terminus (GIMAP6$_{37\text{-}292}$), but not the C-terminus or the G domain of GIMAP6, abrogated the interaction with GIMAP7. Introducing mutations within the G domain of GIMAP6 or truncating the C-terminal 10 amino acids of GIMAP6 can abrogate the interaction with GABARAPL2: however, these variants of GIMAP6 did not appear to have any deficit in their interaction with GIMAP7.

These results indicate that different regions of the GIMAP6 protein are required for the interaction with GIMAP7 and GABARAPL2, respectively (Figure 5.2.10).

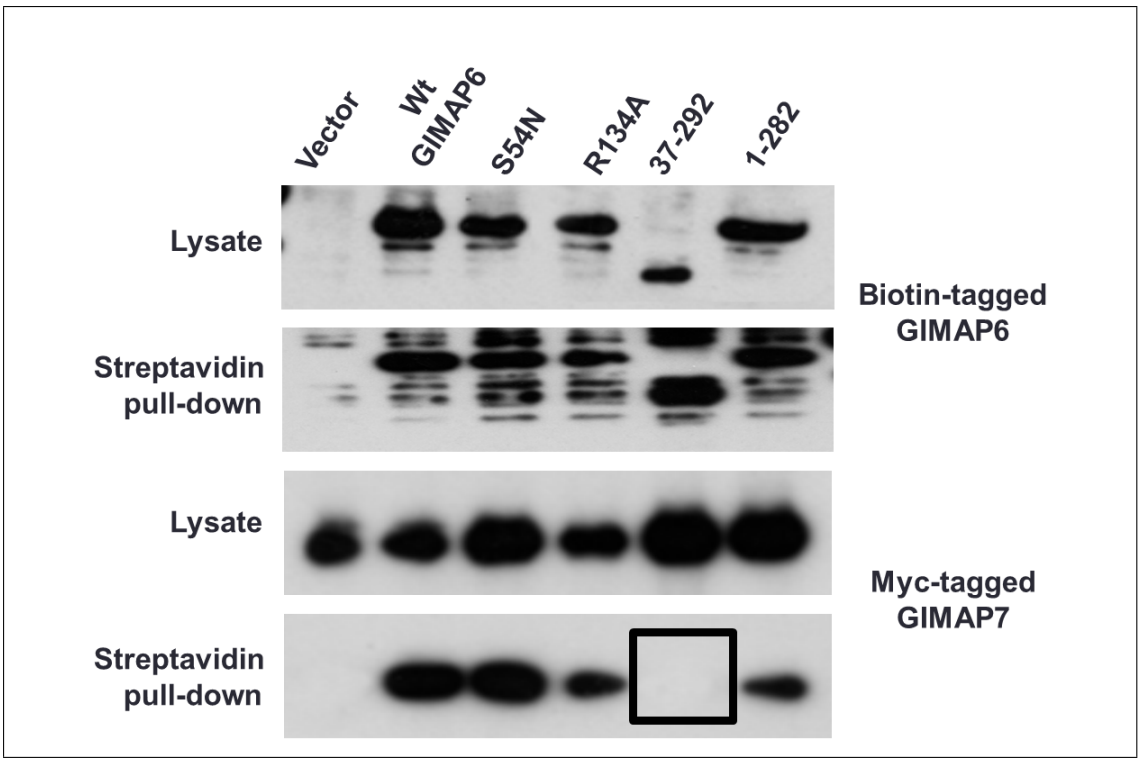

Figure 5.2.10: The N-terminal region of GIMAP6 is required for the interaction with GIMAP7.
HEK293T cells were transfected with a plasmid encoding myc-GIMAP7 together with plasmids encoding biotinylated forms of wt GIMAP6 or the biotinylated GIMAP6 variants as indicated. Cell lysates were prepared and biotinylated and associated proteins recovered by streptavidin-agarose affinity chromatography. Western blots were probed with either the streptavidin-HRP conjugate to detect biotinylated GIMAP6 or the anti-myc monoclonal antibody 9E10 followed by an HRP-conjugated goat anti-mouse IgG to detect myc-tagged GIMAP7.

6 Discussion and Outlook

6.1 Summary of Results

Studies on the GIMAP family have shown that members of this family play crucial roles in the control of lymphocyte survival, with some members, namely GIMAP1 and GIMAP5, being pro-survival (Hellquist et al., 2007; MacMurray et al., 2002; Schulteis et al., 2008; Saunders et al., 2010; Barnes et al., 2010), and others, namely GIMAP4, being potentially pro-death (Dion et al., 2005; Schnell et al., 2006). Little is known about the molecular mechanisms through which the GIMAPs affect lymphocyte survival. Recent studies have indicated that they may interact with members of the Bcl-2 protein family and that GIMAP5 may exercise its anti-apoptotic properties by stabilising Mcl-1 (Chen et al., 2011). A biochemical approach to identify interacting partners of the GIMAPs was undertaken by our laboratory to extend knowledge of the molecular mechanisms that mediate GIMAP function. This led to the identification of GABARAPL2, a mammalian homologue of the yeast autophagy-related gene Atg8, as a major binding partner of GIMAP6 (Pascall et al., 2013). The primary aim of this thesis has been to analyse this interaction in more detail. I have also identified GIMAP7 as a binding partner for GIMAP6. This interaction which has also been observed, independently and using a different approach, by another laboratory (Daumke group; personal communication), may have significant mechanistic implications for the GIMAP family.

The interaction between GIMAP6 and GABARAPL2 was first detected via cross-linking,

I have demonstrated that the interaction can also be detected without cross-linking and have shown that the interaction between GIMAP6 and GABARAPL2 is direct. By employing site-directed mutagenesis and pull-down assays, I have been able to gain an understanding of the molecular requirements for this interaction. Mutational analyses, including point mutations within, and truncations of, GIMAP6 and GABARAPL2 have revealed a number of things. The N-terminal region of GIMAP6, which includes a putative AIM, was not required for the interaction. This was interesting as AIMs are frequently found in proteins that interact with Atg8 family members. However, point mutations within the AIM docking site of GABARAPL2 did disrupt the interaction implying that there could be an as yet unidentified, non-canonical AIM within GIMAP6. Interestingly, the N-terminal region of GIMAP6 *was* required for its interaction with GIMAP7 as its truncation abrogated the ability of GIMAP6 to bind GIMAP7. The GIMAP6-GABARAPL2, but *not* the GIMAP6-GIMAP7 interaction, was disrupted by deleting the last 10 amino acids of GIMAP6, a region predicted to form an α-helix. The GIMAP6-GABARAPL2 interaction was also abrogated when the putative GTPase domain of GIMAP6 was disrupted by mutagenesis. However, interestingly, introducing a point mutation within the GTP binding domain of GIMAP6 had no apparent effect on its interaction with GIMAP7.

To date, no studies have been published on the GTP binding ability of GIMAP6 and I conducted experiments to throw some light on the nucleotide binding status of GIMAP6. While purified GIMAP6 failed to bind isotope-labelled GTP to a detectable level, I have shown for the first time that GIMAP6 expressed in mammalian cells can bind GTP-agarose and that this interaction can be competed by the addition of free GTP and GDP, but not ATP. Indeed, variants of GIMAP6 that could not bind GTP-agarose could also not interact with GABARAPL2, indicating that nucleotide binding may play a crucial role in the interaction.

The crystal structure of GABARAPL2 shows the presence of two α-helices within its N-terminal region (Paz et al., 2000), and deletion experiments demonstrated that the first

α-helix was not only required for the interaction with GIMAP6, but also proved to be sufficient in reproducing the interaction when transplanted into a second Atg8 family member (MAP1LC3B). This implies that the specific binding of GIMAP6 to GABARAPL2 may be due to an interaction between the putative α-helix within the C-terminal region of GIMAP6 and the α-helix in the N-terminal region of GABARAPL2.

Like MAP1LC3B, GABARAPL2 is believed to dock on the autophagosomal membrane through its conjugation to the lipid PE which occurs via a conserved glycine residue present towards its C-terminus. Substituting this residue with alanine did not affect the binding to GIMAP6 which may have implications on the intracellular localisation of the interaction. GABARAPL2 is normally largely cytosolic and only relocalises to autophagosomes when autophagy is induced. Mutating the glycine abrogates this relocation (internal communication, Butcher group) and given that GIMAP6 is also, under normal physiological conditions, believed to be cytosolic, a probable scenario is that the GIMAP6-GABARAPL2 interaction occurs in the cytosol. GIMAP6, like GABARAPL2, has been shown in our laboratory to relocate to autophagosomes on the induction of autophagy where it co-localises with both MAP1LC3B and GABARAPL2 (Pascall et al., 2013). In this thesis, I have demonstrated that a mutant GIMAP6 that is unable to interact with GABARAPL2 loses its ability to relocate to autophagosomes and remains cytosolic.

Finally, as has been observed for GIMAP2 and GIMAP7 (Schwefel et al., 2010, 2013), I have observed that GIMAP6 can interact with itself, a property that is shared with septins (de Almeida Marques et al., 2012) and dynamins (Alpadi et al., 2013; Warnock et al., 1996; de Almeida Marques et al., 2012) and is necessary for their ability to function. The significance of this for the biology of GIMAP6 is as yet unclear.

6.2 The N-terminal region of GIMAP6

Apart from mediating membrane fusion (Weidberg et al., 2011), a major role of members of the Atg8 family is recruiting proteins to autophagosomes (Noda et al., 2010). As has been mentioned earlier (subsection 3.1.2), proteins that interact with the Atg8 family commonly do so via the presence of an AIM (W/YXXI/L; (Svenning et al., 2011; Noda et al., 2010)). AIM-containing proteins are usually promiscuous and able to interact with multiple members of the Atg8 family, with the promiscuity down to the presence of the motif. An example of this is NBR1, which has been shown to interact with MAP1LC3B, GABARAP and GABARAPL2 (Lamark et al., 2009; Kirkin et al., 2009a). Indeed, a large-scale analysis found that out of 67 Atg8-interacting proteins identified, 31 interacted with single members of the family, with GABARAPL2 interacting uniquely with only one, WDR62 (Behrends et al., 2010). Intriguingly, while the N-terminal region of GIMAP6 contains the AIM (6YEQI9), our laboratory has demonstrated that GIMAP6 is specific for GABARAPL2, amongst Atg8 family members, which is rather unusual (Pascall et al., 2013). Mutational (Figure 3.3.3) and truncation studies (Figure 3.3.14) suggested that the AIM motif within GIMAP6 did not have an obvious role in the GIMAP6-GABARAPL2 interaction. The high specificity of the interaction may be consistent with the apparent non-involvement of the putative AIM within GIMAP6. Recent work on the Atg19-Atg8 interaction has suggested a similar phenomenon where the presence of an AIM within Atg19 does not appear to play an important role in the interaction (Sawa-Makarska et al., 2014).

Based on results obtained when the putative AIM within GIMAP6 was mutated and deleted, I hypothesised that the residues within the ADS of GABARAPL2 would not be involved in the GIMAP6-GABARAPL2 interaction. Unexpectedly, mutations within the ADS of GABARAPL2 resulted in a complete loss of interaction with GIMAP6- (Figure 4.3.3), indicating that the GIMAP6-GABARAPL2 interaction may still involve

AIM-ADS interactions, albeit with the AIM within GIMAP6 possibly being non-canonical and as yet unidentified. Interestingly, recent work has demonstrated the existence of non-canonical AIMs (von Muhlinen et al., 2012; Kaufmann et al., 2014; Newman et al., 2012). von Muhlinen et al. identified that NDP52 is specific for MAP1LC3C and binds to it by virtue of a variant AIM comprising the sequence ILVV. However, this interaction takes place in a manner that bypasses docking of the hydrophobic residues of the AIM (at positions 1 and 4) in the hydrophobic pockets of MAP1LC3C. A similar motif within the GIMAP6 amino acid sequence was not identified and, furthermore, it is unlikely for GIMAP6 to have an AIM that functions in a manner analogous to the one seen in NDP52 as mutations within the hydrophobic pocket abrogated the GIMAP6-GABARAPL2 interaction. By analysing the structure of the Atg12-Atg5 complex solved by Otomo et al. (2013), Kaufmann et al. 2014 demonstrated the existence of a non-canonical, non-contiguous, three-dimensional AIM in Atg12 involving Trp139 and Val62, with this motif proposed to be required for the Atg8-dependent recruitment of the Atg12–Atg5–Atg16 complex to the phagophore subsequent to the Atg12–Atg5–dependent conjugation of Atg8. They observed that these residues were positioned such that their Cα atoms matched the critical distance of 9.8 Å, a distance that also separates the conserved residues W/YXXI/L in canonical AIMs. Whether the AIM found in Atg12 makes contact with the hydrophobic pockets of Atg8 is unknown, but if that were to be the case the determination of the structure of GIMAP6 may aid in the identification of the proposed AIM within its sequence.

While the N-terminal region of GIMAP6 did not have a role to play in the interaction with GABARAPL2, interestingly, truncating it abrogated the interaction with GIMAP7 (Figure 5.2.10). Further work is necessary to identify the residues within the N-terminal region of GIMAP6 that are required for its interaction with GIMAP7, and to identify regions and residues within GIMAP7 that play a role in the GIMAP6-GIMAP7 interaction.

6.3 The C-terminal region of GIMAP6 and the N-terminal region of GABARAPL2

The region C-terminal to the GTPase/AIG1 domain of GIMAP6 (amino acids 243-292) is predicted to contain two α-helical extensions by PSIPRED, a secondary structure prediction programme (Buchan et al., 2013; Jones, 1999; Figure 6.3.1, C-terminal helices highlighted in green). As mentioned previously (subsection 4.1.1), the N-terminal region of GABARAPL2 contains two α-helices (Paz et al., 2000). α-helices comprise approximately 40% of all secondary structures (Cummings and Hamilton, 2010) and are frequently found at the interface of protein-protein interactions (PPI). Truncating the putative α-helices within the C-terminal region of GIMAP6 abolished the GIMAP6-GABARAPL2 interaction (Figure 3.3.14). Point mutations were introduced within this region and the results I obtained indicated an important role for R284 within GIMAP6 as substituting this residue with an alanine almost completely abrogated the interaction with GABARAPL2. This is consistent with the finding that arginines and aromatic residues are over-represented as hot spots at helical interfaces (Bullock et al., 2011; Azzarito et al., 2013). The results also indicated that the cumulative character of the terminal 10 amino acids within GIMAP6 was critical for the interaction with GABARAPL2. This region contains a mixture of both charged and hydrophobic residues - HRCLLGKADL (in colour, polar; in blue, positively charged; in red, negatively charged; other, hydrophobic).

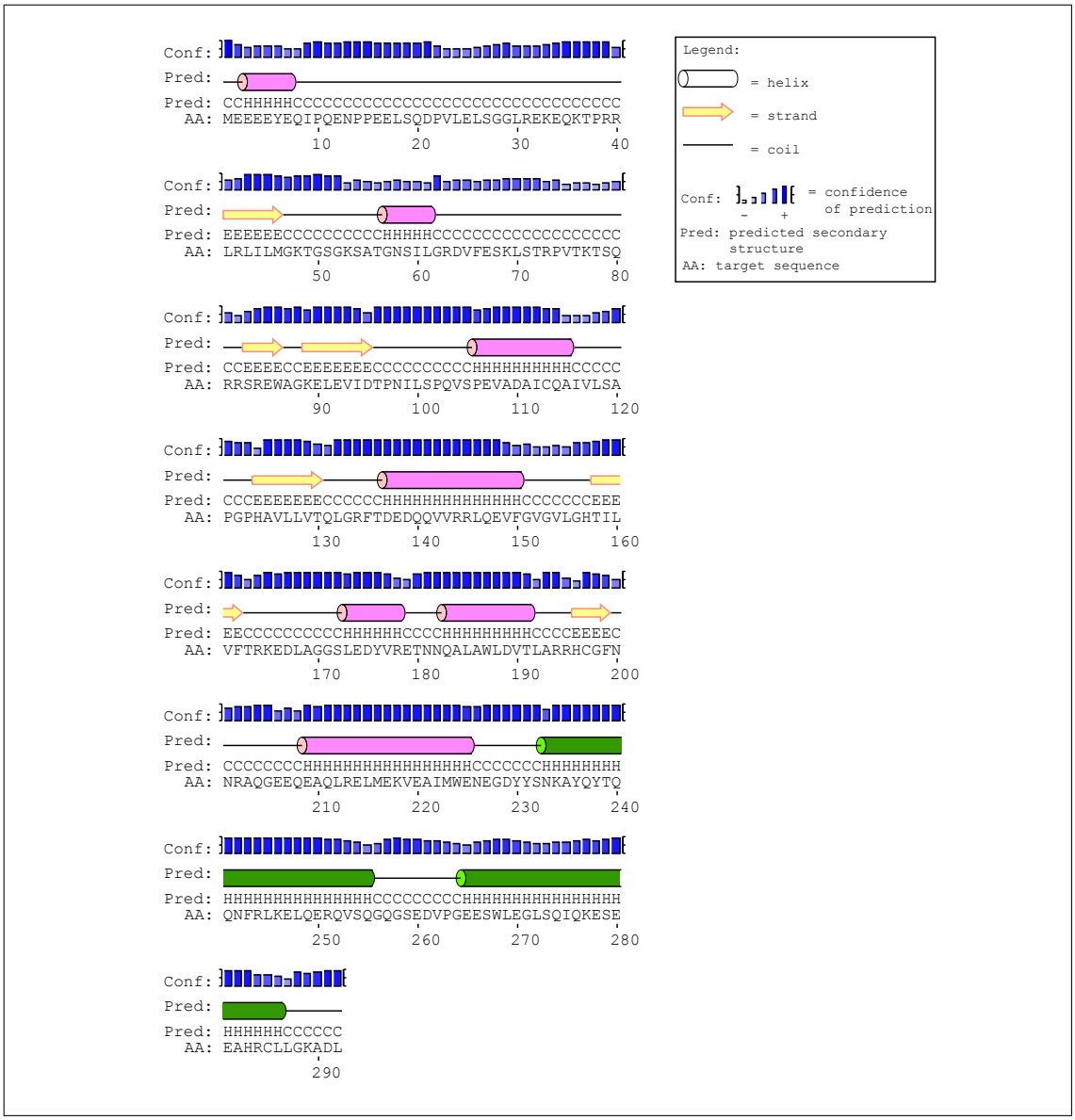

Figure 6.3.1: GIMAP6 secondary structure prediction.
Cylinders in green represent the helices within the C-terminal region of GIMAP6.

An interesting observation was that truncating the C-terminal region within GIMAP6 also adversely affected GTP binding activity (Figure 3.3.16), possibly implying that this region somehow plays a role in maintaining the structure of GIMAP6 such that it is able to have a functioning GTP binding domain. Indeed, structural analysis of GIMAP2 indicated that the α-helix within its C-terminal extension folded against its G domain (Schwefel et al., 2010). Alternatively, as alluded to above, the C-terminal region of GIMAP6 may be dir-

ectly involved in making contact with the N-terminal region of GABARAPL2. Disrupting this interaction may ultimately, through as yet unknown mechanisms, disrupt the ability of GIMAP6 to bind GTP.

Although the GTPase/AIG1 domain amongst the various mammalian GIMAP6 orthologues is well conserved there is more divergence outside of that region. Interestingly, three rodent species (mouse, rat and chinese hamster), but not the mole rat, have truncated C-terminal domains compared with several other mammalian species, including humans. Mouse GIMAP6 (mGIMAP6) did not, in my hands, interact with GABARAPL2, consistent with importance of the C-terminal region for the interaction. Attempts were made to replace the C-terminal region of mGIMAP6 with the corresponding region of hGIMAP6 and, separately, to fuse this region to the C-terminal end of GFP to analyse whether this region alone would reproduce the interaction with GABARAPL2. However, unfortunately, I was unable to obtain functioning constructs. A variety of different cloning protocols were employed to no avail.

As mentioned in subsection 4.1.1, the presence of two α-helices at the N-terminus of Atg8 homologues is well conserved (Weiergraber et al., 2013); however, the character of these helices is not (this region within the MAP1LC3 subfamily is primarily composed of positively charged amino acids, while in the GABARAP subfamily the region is composed of hydrophobic residues). This may have implications with respect to the stage of autophagosome biogenesis at which MAP1LC3B and GABARAPL2 are involved (Weidberg et al., 2010). While the MAP1LC3 subfamily is involved in elongation of the phagophore membrane, the GABARAP subfamily is essential for the sealing of the autophagosomes and, the difference in the helices may affect the nature of the interaction with the membrane, leading to differing outcomes. The difference in the helices also affects the ability to bind SQSTM1, an autophagic cargo receptor implicated in autophagy-dependent elimination of cytosolic ubiquitinated proteins, organelles such as mitochondria and peroxisomes, and bacteria (Komatsu and Ichimura, 2010). While both MAP1LC3B and

GABARAPL2 can bind SQSTM1, only MAP1LC3B can deliver SQSTM1 to autolysosomes and this difference in the behaviour of the two proteins was pinpointed to the differences in the N-termini of MAP1LC3B and GABARAPL2 (Shvets et al., 2011).

I swapped the first α-helix within the N-terminal region of MAP1LC3B with the corresponding region of GABARAPL2 (comprising the amino acids MKWMFKEDHS; refer to key above) and demonstrated that the chimeric protein was able to interact with GIMAP6 (Figure 4.3.2). Unlike SQSTM1 which is able to interact with both GABARAPL2 and MAP1LC3B in the cytosol but only associates with membrane-bound MAP1LC3B and not membrane-bound GABARAPL2 (Shvets et al., 2011); GIMAP6 appears to be unable to interact with either form of MAP1LC3B. The N-terminal region of GABARAPL2 thus plays a critical role in the interaction with GIMAP6, however, it is not yet possible to comment on whether this helix alone is sufficient to mediate the interaction. As mentioned above, residues within the ADS of GABARAPL2 appear to be necessary for the interaction with GIMAP6; this region is also present in MAP1LC3B and a potential future experiment would be to analyse if the chimeric MAP1LC3B with point mutations within its ADS was still able to interact with GIMAP6. Lystad et al., 2014, have recently identified three residues, K24/Y25/D54, within the GABARAP subfamily that are responsible for the selective binding of ALFY (autophagy-linked FYVE protein, also called WDFY3), a large phosphatidylinositol 3-phosphate-binding protein shown to be recruited to ubiquitin-positive structures during stress that interacts with the ubiquitin-binding autophagy receptors SQSTM1 and NBR1 and contributes to autophagic clearance of aggregated proteins (Filimonenko et al., 2010; Clausen et al., 2010). Whether these residues within GABARAPL2 are responsible for the specificity of the interaction with GIMAP6 is a potential avenue for future research.

Interestingly, the 10 C-terminal amino acids of GIMAP6 did not appear to be required for the interaction with GIMAP7 (Figure 5.2.10). The C-terminal truncate of GIMAP6 was unable to bind both GTP and GABARAPL2, however, the interaction with GIMAP7

remained unaffected. This may indicate that the GIMAP6-GIMAP7 interaction could be independent of the activity of the GTP binding/AIG1 domain of GIMAP6. Alternatively, GIMAP6 may adopt a conformation that allows its N-terminal region to make contact with GIMAP7, while, simultaneously, the C-terminal region adopts a conformation such that the putative GTP binding region of GIMAP6 remains active allowing it to bind GABARAPL2. Cross-linking studies indicated that GIMAP6 and GABARAPL2 formed a complex, but this could be due to the particular spatial arrangement of a hypothetical GIMAP6-GABARAPL2-GIMAP7 complex that precluded the identification of GIMAP7 as an interacting partner via the cross-linking methodology.

GIMAP6, like GIMAP2 and GIMAP7, can interact with itself (Figure 5.2.7). GIMAP2 homodimerises to prevent the catalysis of GTP hydrolysis and heterodimerises with GIMAP7 to catalyse GTP hydrolysis (Schwefel et al., 2010, 2013). Whether the GIMAP6-GIMAP6 interaction has a role in sequestering it from both GABARAPL2 and GIMAP7 is unknown and the physiological conditions under which these interactions occur need investigating.

Whether mouse GIMAP6 interacts with mouse GIMAP7 (or the closely related mouse GIMAP9) is currently unknown and would be interesting to investigate. Furthermore, given that hGIMAP proteins exhibit intra-family interactions (this thesis and Schwefel et al., 2013; Schwefel and Daumke, 2011), it will be interesting to test whether the mGIMAPs display similar behaviour. These experiments would provide valuable insight into the biology of the GIMAP family of proteins.

6.4 The GTPase domain of GIMAP6

Of the hGIMAP proteins, until recently the GTP binding and hydrolytic ability of only hGIMAP4 had been characterised (Cambot et al., 2002). Recent work by the Daumke group, a structural biology laboratory that studies the GIMAP family of proteins, has

thrown light on the GTP binding and hydrolytic activities of hGIMAP2 (Schwefel et al., 2010) and hGIMAP7 (Schwefel et al., 2013). Nothing about the GTP binding status or hydrolytic activity of hGIMAP6 was known prior to my attempts to address these questions. Unfortunately, technical issues related to the purifying of GIMAP6, hindered my attempts to characterise the GTP binding and hydrolytic abilities of GIMAP6. An attempt was made to purify GST-tagged GIMAP6 that was bacterially expressed; however, it proved very hard to obtain soluble GST-GIMAP6 that could be purified and employed in assays with isotope-labelled GTP. Bacteria were induced at low temperature, and lysed, in either the presence and absence of GDP, albeit without any positive effect on its solubility. Assays with [^{35}S]GTPγS were carried out both with GST-GIMAP6 that was immobilised on glutathione beads and with GST-GIMAP6 that had been eluted off the beads. They yielded results saying that the protein preparation did not bind GTP, either because that is the intrinsic property of GIMAP6 in isolation or because the preparation was in some way denatured. A literature review was carried out and an alternate method to answer the question of whether GIMAP6 could bind GTP/GDP was employed.

The use of GTP-agarose beads has been used to analyse the GTP binding ability of various GTPases (Pendin et al., 2011; Modiano et al., 2005; Jebelli et al., 2012; Carlessi et al., 2011) and was used in this study to investigate the nucleotide binding ability of GIMAP6. The advantage of this method is that it is possible to assay GTP binding in crude lysates taken directly from living cells. Using this approach, I demonstrated that GIMAP6 was unable to bind ATP but could bind GDP and GTP, with the affinity for GTP being higher than that for GDP, a phenomenon observed in most GTPase families (Bourne et al., 1991). This assay is unable to test the catalytic activity of GIMAP6 and therefore cannot be used to determine whether a variant of GIMAP6 that could not bind to GTP-agarose was unable to do so because it was unable to bind nucleotide at all or because it was inaccessible to GAPs and therefore in a permanently GTP-bound state; the latter would result in awry nucleotide cycling and prevent the variant in question from binding to GTP-agarose. It

may require GIMAP6 to be expressed in (and purified from) an insect or mammalian cell line to overcome solubility issues for assays with [^{35}S]GTPγS or [γ^{32}P]GTP to be feasible.

As detailed in subsection 3.3.4, point mutations within the GTP binding domain of GIMAP6 abrogated the interaction with GIMAP6, with variants unable to bind to GTP-agarose also unable to interact with GABARAPL2. Mutations within the GTP binding domain of GIMAP6 had been made on the basis of predictions made when similar mutations had been studied in the Ras family of GTPases. This was the first opportunity to test their validity in the GIMAPs. With the exception of D167A within the G4 motif - (subsection 3.3.4), mutations within the other motifs within the GTP binding/AIG1 domain of GIMAP6 could abrogate interaction with GABARAPL2. It would be interesting to check the ability of this variant of GIMAP6 to bind to GTP-agarose.

It is worth considering whether GIMAP6's interaction partner GABARAPL2 shows any characteristics of a classical GAP or GEF. If GABARAPL2 was an effector of GIMAP6, then one could reasonably predict the GIMAP6$_{G50D}$ variant to be constitutively bound to GABARAPL2, an interaction that has been observed with other GTPases and their effectors (reviewed in Cherfils and Zeghouf, 2013 and Schubbert et al., 2007). Alternatively, the putative constitutively active variant of GIMAP6 could be resistant to GAPs, a role that may be played by GABARAPL2. GTP hydrolysis assays in the presence and absence of GABARAPL2 are necessary to test this hypothesis. GABARAPL2 was first identified as an ATPase enhancer (Muller et al., 2002; Sagiv et al., 2000 refer section 4.1), and it may play an analogous role in the context of GIMAP6. Dominant-negative GTPases are thought to be unable to bind GTP (Itzen et al., 2006; McMahon, 2004), and exhibit a higher affinity for GEFs (reviewed in Cherfils and Zeghouf, 2013 and Heasman and Ridley, 2008). The putative dominant-negative GIMAP6 variant, GIMAP6$_{S54N}$ was unable to bind GABARAPL2, however, making it unlikely that GABARAPL2 acts as a GEF for GIMAP6. It could be that nucleotide exchange is key for the ability of GIMAP6 to bind

GABARAPL2 and to relocate to autophagosomes. A somewhat analogous phenomenon was observed with the targeting of human GBP-1 to the Golgi. hGBP-1 can target specifically to the Golgi membrane in its GTP-bound form; non-hydrolysable GTP bound mutants are unable to do so, however, suggesting that nucleotide exchange may be a significant factor in hGBP-1 localisation (Modiano et al., 2005). Interestingly, GIMAP6$_{S54N}$ was still able to interact with GIMAP7, indicating that GIMAP6 may not require an intact GTP binding capacity for binding GIMAP7 (Figure 5.2.10). Further interaction studies with other variants of GIMAP6 need to be carried out for a more detailed understanding of this interaction.

The arginine at position 117 within GIMAP2 (this arginine is conserved across all GIMAP family members) is required for dimerisation and GTP binding (Schwefel et al., 2010). Mutating the corresponding residue in GIMAP6 (R134), however, did not have any apparent effect either on its GTP binding (Figure 3.3.11), on its ability to bind GABARAPL2 (Figure 3.3.10) or with GIMAP7 (Figure 5.2.10). This is somewhat analogous to what has been observed in GIMAP7, where mutating the arginine does not inhibit the dimerisation or GTP binding ability of GIMAP7; however, this residue was shown to be necessary for GIMAP7's GAP activity, both for itself and for GIMAP2 (Schwefel et al., 2013). Does GIMAP6 exhibit GAP activity for other members of the GIMAP family? This would be an interesting question to work on. Interestingly, R117 within GIMAP2 makes contact with the Q114 in the opposing GIMAP2 monomer and mutating the Q114 abrogated GIMAP2 homodimerisation. A similar scenario may exist in GIMAP6, and substituting the Q131 within GIMAP6 may affect its apparent oligomerisation. Neither truncating the N-terminal region of GIMAP6, nor truncating the C-terminal region hampered the ability of GIMAP6 to interact with itself. This may indicate that the GTP binding domain within GIMAP6 may have a role to play in the self-interaction, however, the dominant-negative mutant of GIMAP6 was also able to self-interact subsection 5.2.3 and Figure 3.3.10. Analysing further variants of GIMAP6 with mutations in its GTP binding domain may be

helpful. Additionally, residues predicted to be involved in aggregation are represented in (Figure 6.4.1) and introducing mutations within this region may affect the ability of GIMAP6 to interact with itself.

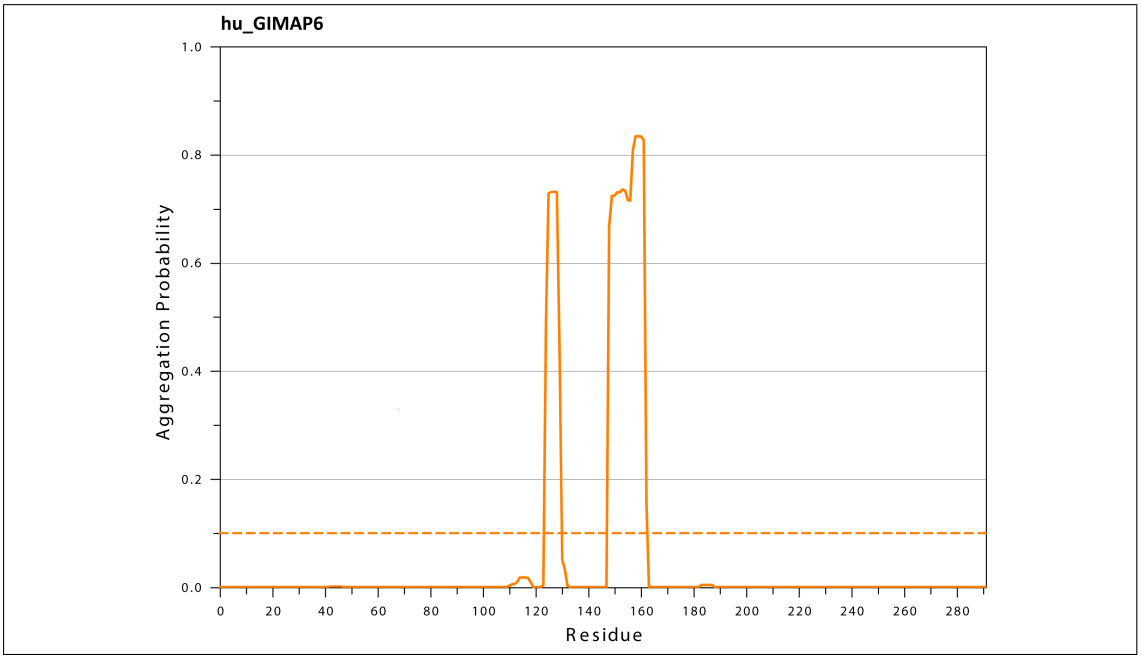

Figure 6.4.1: Regions within GIMAP6 that may be involved for aggregation. The peaks represent the regions within GIMAP6 that may be involved in it's aggregation. The prediction is based on DisEMBL, a computational tool used to predict disordered/unstructured regions within a protein sequence Linding et al. (2003).

It may be that the structural conformation adopted by GIMAP6 with an active nucleotide (GTP) exchange capacity is key to its interaction with GABARAPL2. Solving the structure of GIMAP6 will aid in better understanding the GIMAP6-GABARAPL2 and GIMAP6-GIMAP7 interactions. Technical issues with regards to the solubilisation of GIMAP6 may be circumvented by co-purifying the GIMAP6-GABARAPL2 or the GIMAP6-GIMAP7 complex or the use of antibody Fab fragments and then attempting structural studies.

6.5 Functional significance of the GIMAP6-GABARAPL2 interaction

The GABARAP subfamily of which GABARAPL2 is a member has been proposed to be involved in the latter stages of autophagosome biogenesis (Weidberg et al., 2010), and the discovery of GIMAP6 as a seemingly quantitatively major binding partner of GABARAPL2, along with the relocation of both proteins to autophagosomes in response to cell starvation, suggests a potential role for GIMAP6 in the autophagic process. GTPases and GTPase-associated proteins have been shown to play active roles in various aspects of membrane (reviewed in Burd and Collins, 2004; Lamb et al., 2013; Nachury et al., 2007) and autophagosome biogenesis (Itoh et al., 2011; Popovic et al., 2012; Yamano et al., 2014). GIMAP6, too, could have a role to play in autophagosome membrane elongation, for instance in a manner similar to the interaction between Rab33-GAP, OATL1, and Atg8 family members (Popovic et al., 2012), it is possible that GABARAPL2 could serve as a scaffold from which GIMAP6 may perform as yet unidentified functions within the autophagic pathway. Our laboratory has investigated whether modulating levels of GIMAP6 could influence the autophagic process, however, no simple relationship to the process of autophagy as defined by the conversion of MAP1LC3B-I to MAP1LC3B-II, or a shift in the number of MAP1LC3B positive punctae (a classic autophagosomal marker) was observed (Pascall et al., 2013). I asked whether the GIMAP6-GABARAPL2 interaction was necessary for the relocation of GIMAP6 to autophagosomes to perhaps permit either its selective degradation or to facilitate a specific function at the vesicles. I found that variants of GIMAP6 that failed to interact with GABARAPL2 were not recruited to autophagosomes. While it may be that GIMAP6 interacts with other proteins that mediate its recruitment to autophagosomes, it seems likely that the GIMAP6-GABARAPL2 interaction has a key role in the recruitment. A possible strategy to add further clarity to this particular question could be to conduct experiments with cell lines that preferably

express GIMAP6 endogenously and either do not express GABARAPL2, or are engineered to express GABARAPL2 at reduced levels. The imaging experiments were carried out with engineered HEK293 cells due to the difficulties associated with imaging Jurkat T cells which do express GIMAP6 endogenously.

Studies in our laboratory have indicated that the recruitment of GIMAP6 to autophagosomes may be associated with its turn-over. These experiments were carried out using Jurkat T cells and the endogenous protein levels were analysed. GIMAP6 has been shown to be able to regulate cellular GABARAPL2 levels (Pascall et al., 2013). Mutating G116 within GABARAPL2, a residue that is critical for its lipidation and subsequent membrane docking, did not affect its binding to GIMAP6, indicating that this interaction can occur in the cytosol. I have demonstrated that the interaction is direct (Figure 5.2.1) and have obtained very preliminary data indicating that these proteins may be able to exist as a complex *in vivo* (Figure 5.2.6). Unfortunately, I was unable to obtain this result on a consistent basis: different lysis buffers, and lysis buffer concentrations were trialled to no avail and induction of autophagy also did not appear to have any effect (not shown). The positive experiment performed Figure 5.2.6 was controlled internally as far as was possible, and it may be that the complex is unstable and needs a very particular set of circumstances to enable one to detect it.

Given that GIMAP6 can regulate GABARAPL2 protein levels, is GIMAP6 being sequestered by GABARAPL2 in the cytosol (or *vice versa*), in a manner similar to when Cdc42 is sequestered in the cytosol by its guanine nucleotide dissociation inhibitor (GDI) (a molecule that maintains GTPases in the GDP bound state) (Dirac-Svejstrup et al., 1997; Iden and Collard, 2008; Forget et al., 2002)? On a somewhat related note, a preliminary examination of cell lines engineered to express biotinylated GIMAP6 with mutations inserted within the G1 motif (G50D and S54N) indicated that neither cell line was able to express the stably transfected protein to any detectable level. Could this be a result of the inability of these variants to bind GABARAPL2 and thereby be protected

from degradation? Stabilising GIMAP6 might facilitate its function if GABARAPL2 could dissociate adequately. Alternatively, the potential sequestration of GIMAP6 by GABARAPL2 could prevent GIMAP6 from performing functions either related to autophagy or not, and possibly in conjunction with GIMAP7. Does it require a particular physiological event for the GIMAP6-GABARAPL2 interaction to be disrupted to then allow the GIMAP6-GIMAP7, or GIMAP6-GIMAP6 interactions, amongst other as yet undiscovered interactions? GABARAPL2, too, may have other functions in the cell besides taking part in the autophagosome biogenesis and further studies on GABARAPL2, particularly in relation to a knock-out animal model would be informative. As has been alluded to above, the mouse and the rat have truncated C-terminal domains (Figure 3.3.13) and this may be why no interaction between the mouse GIMAP6 and GABARAPL2 is observed. So, while these may not be the best animal models to study the significance of the GIMAP6-GABARAPL2 interaction, it must be noted that all other mammalian GIMAP6 sequences examined do not have similar truncations and may be able to interact with GABARAPL2 (Figure 3.3.13). This suggests that the modification seen in muroid rodents may be relatively confined taxonomically. This, along with the specificity of the GIMAP6-GABARAPL2 interaction implies a potentially fundamental role and the interaction needs further investigating.

6.6 Functional significance of the GIMAP6-GIMAP7 interaction

GIMAP2 has been shown to associate with both itself and GIMAP7 and while GIMAP2 has no significant hydrolytic activity of its own (Schwefel et al., 2010), GIMAP7 can act as a GAP for both itself and for GIMAP2 (Schwefel et al., 2013). On the basis of these findings, I asked whether GIMAP6 was able to interact with other members of the GIMAP family and discovered that it could interact with GIMAP7 (Figure 5.2.9).

For members of the Ras superfamily, GTP hydrolysis is stimulated by association with GTPase-activating proteins (GAPs) which often supply a catalytic arginine residue (Bos et al. 2007b; subsection 1.3.1). Studies on other families of GTPases have demonstrated that GTPase activity can be triggered by nucleotide-dependent dimerisation of the G domains. Within the IRGs, for instance, interactions between members of two functionally distinct subgroups have been shown to modulate catalytic activity (Hunn et al., 2008). Unlike what has been observed with GIMAP2 and GIMAP7, members of one IRG subgroup assemble with proteins of the second subgroup to prevent GTP loading and activation, with homodimerisation being required for the catalytic mechanism (Pawlowski et al., 2011). Heterodimer formation and subsequent GTPase activation is also observed in the signal recognition particle (SRP) and its receptor (SRPR), septins, septin-related Toc proteins, dynamins, and the Roco protein Leu-rich repeat kinase 2 (LRRK2) (Gasper et al., 2009; Sun et al., 2002; Praefcke and McMahon, 2004; Sirajuddin et al., 2007; Grudnik et al., 2009). In the case of the GIMAPs, work has shown that GTPase activity is stimulated by dimerisation, with the conserved arginine from the conserved box motif having a dual function: it plays a role in the homodimerisation of GIMAP2 and within GIMAP7 is able to act as a catalytic arginine finger to stimulate GTP hydrolysis in both GIMAP2 and GIMAP7 (Schwefel et al., 2010; Schwefel and Daumke, 2011; Schwefel et al., 2013). Interestingly, however, in my experiments mutations of this arginine within GIMAP6 did not reveal a role in either homodimerisation (Figure 5.2.8) or heterodimerisation (Figure 5.2.10) and its role in GIMAP6 biology needs further investigation. What role does the GIMAP6-GIMAP7 interaction have? I postulate that GIMAP7 acts as a GAP for GIMAP6. Given that it is able to act as a GAP for both itself and for GIMAP2, it is plausible that GIMAP7 acts as a GAP activity for the GIMAP family and investigations into whether other members of the GIMAP family can interact with GIMAP7 will be interesting. Sub-cellullar localisation studies to investigate whether GIMAP6 co-localises with GIMAP7 or whether it is able to do so under certain physiological conditions will

also be informative.

6.7 Concluding Remarks

Members of the GIMAP family have opposing functions in lymphocyte survival. For instance, GIMAP1 and GIMAP5 are believed to be pro-survival, while GIMAP4 is believed to accelerate T cell death (Chen et al., 2011; Schulteis et al., 2008; Barnes et al., 2010; Schnell et al., 2006; Saunders et al., 2010). One could envision a scenario where GIMAP members act as sensors within the lymphocyte and are able to trigger apoptosis under certain conditions. Members with differing functions might interact with one another and somehow (possibly via GTP binding and hydrolysis) regulate the pro- and anti-apoptotic function/s of their partners. The GIMAPs could then be seen as an interacting network or system. While studies to date have by and large implicated the GIMAPs in adaptive immunity, they may also have roles to play in innate immunity; plant homologues of the GIMAPs have been shown to be induced when challenged by bacterial (Reuber and Ausubel, 1996) and fungal infections (Liu et al., 2008) and a recent study has demonstrated that GIMAPs were strongly up-regulated within corals when these were bacterially challenged (Weiss et al., 2013).

The dynamin-like IRG family of proteins is a pertinent example. IRGs respond to intracellular pathogens and the various family members act in concert to deliver phagocytosed pathogens for clearance via autophagy. Mouse IRGs can be divided into two subgroups based on the presence of a canonical lysine or a non-canonical methionine within the G1 motif (GxxxG[K/M]S/T). The GMS proteins act as regulators of the nucleotide-bound state of the GKS proteins, helping to control their GTP-dependent activation. In turn, this controls the antimicrobial activities of their respective effectors which can include other GKS-containing IRGs and proteins involved in autophagy (Taylor et al., 1996; Tiwari et al., 2009; Kim et al., 2012; Hunn et al., 2008). Furthermore, the human IRGM protein

is interesting as, in addition to playing a role in innate cell defense, it also plays a role in cell survival through autophagy and, by localising to the mitochondria and affecting mitochondrial fission it is also involved in apoptosis. GIMAPs can also be subdivided into two groups (albeit based on the ability to be membrane-bound or not): membrane-anchored GIMAPs possessing a C-terminal hydrophobic segment (hGIMAP1, 2, and 5) and soluble GIMAPs (4, 6, 7 and possibly 8) which do not have such a hydrophobic stretch. Data on the GIMAPs so far suggest that membrane-anchored GIMAPs form nucleotide-regulated oligomers on the surface of cellular organelles. These might function in a manner similar to the septin oligomers (Weirich et al., 2008; Sirajuddin et al., 2007) and, amongst other roles, they could organise the assembly of interaction partners in a manner that is analogous to 'septin cages' which compartmentalise pathogens within the cell and eliminate them via autophagy (reviewed in Mostowy and Cossart, 2011).

The precise significance of the GIMAP6-GABARAPL2 and GIMAP6-GIMAP7 interactions remains to be elucidated . Experiments that add further clarity to the GTP binding and hydrolysis of the GIMAP members will be key. Studying GIMAP6- and GIMAP7-deficient mice may also help in the understanding of GIMAP biology and studies that examine whether the mammalian GIMAPs have a role to play in innate immunity would seem to be justified.

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